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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 39/395 // C07K 16/28, 16/46

A2

(11) International Publication Number: **WO 98/47531**

(43) International Publication Date: 29 October 1998 (29.10.98)

(21) International Application Number: PCT/US98/08029

(22) International Filing Date: 21 April 1998 (21.04.98)

(30) Priority Data:
60/044,084 21 April 1997 (21.04.97) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 60/044,084 (CIP)
Filed on 21 April 1997 (21.04.97)

(71) Applicant *(for all designated States except US)*: ARCH DEVELOPMENT CORPORATION [US/US]; 1101 East 58th Street, Chicago, IL 60637 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: SMITH, Judith, A. [US/US]; 1606 E. 50th Place, Apt. 4C, Chicago, IL 60615 (US). TSO, J., YunCLARK, Marcus, R. [US/US]; 5841 S. Maryland, MC0930, Chiacgo, IL 60637 (US). COLE, Michael, S.BLUESTONE, Jeffrey, A. [US/US]; 5421 S. Kenwood, Chicago, IL 60615 (US).

(74) Agent: WILSON, Mark, B.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: FC RECEPTOR NON–BINDING ANTI–CD3 MONOCLONAL ANTIBODIES DELIVER A PARTIAL TCR SIGNAL AND INDUCE CLONAL ANERGY

(57) Abstract

Anti–CD3 mAbs are potent immunosuppressive agents used in clinical transplantation. However, the activation–related adverse side effects associated with these mAbs have prompted the development of less toxic Fc receptor non–binding anti–CD3 mAb therapies. At present, the functional and biochemical consequences of T cell exposure to Fc receptor non–binding anti–CD3 is unclear. In this study, the inventors have examined the early signaling events triggered by a Fc receptor non–binding anti–CD3 mAb. Like the mitogenic anti–CD3 mAb, Fc receptor non–binding anti–CD3 triggered changes in the TCR complex, including ζ chain tyrosine phosphorylation and ZAP–70 association. However, unlike the mitogenic anti–CD3 stimulation, Fc receptor non–binding anti–CD3 was ineffective at inducing the highly phosphorylated form of ζ (p23) and tyrosine phosphorylation of the associated ZAP–70 tyrosine kinase. This proximal signaling deficiency correlated with minimal PLCγ–1 phosphorylation and failure to mobilize detectable Ca^{2+}. Not only did biochemical signals delivered by Fc receptor non–binding anti–CD3 resemble altered peptide ligand signaling, but exposure of Th1 clones to Fc receptor non–binding anti–CD3 also resulted in functional anergy. Finally, a bispecific anti–CD3 x anti–CD4 $F(ab)'_2$ reconstituted early signal transduction events and induced proliferation, suggesting that defective association of lck with the TCR complex may underlie the observed signaling differences between the mitogenic and Fc receptor non–binding anti–CD3.

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DESCRIPTION

FC RECEPTOR NON-BINDING ANTI-CD3 MONOCLONAL ANTIBODIES DELIVER A PARTIAL TCR SIGNAL AND INDUCE CLONAL ANERGY

BACKGROUND OF THE INVENTION

The present application is a continuation–in–part of co–pending U.S. Provisional Patent Application Serial No. 60/044,084 filed April 21, 1997. The entire text of the above–referenced disclosure is specifically incorporated by reference herein without disclaimer.

1. Field of the Invention

This invention relates generally to methods and materials for modulation of the immunological activity and toxicity of immunosuppressive agents derived from murine OKT3 used in organ transplantation and in the treatment of auto-immune diseases.

2. Description of Related Art

OKT3 is a murine monoclonal antibody (mAb) which recognizes an epitope on the ε-subunit within the human CD3 complex (Salmeron, 1991; Transy, 1989; *see also*, U.S. Patent No. 4,658,019, herein incorporated by reference). Studies have demonstrated that OKT3 possesses potent T cell activating and suppressive properties depending on the assay used (Landgren, 1982; Van Seventer, 1987; Weiss, 1986). Binding of OKT3 to the TcR results in coating of the TcR and or modulation, thus mediating TcR blockade, and inhibiting alloantigen recognition and cell-mediated cytotoxicity. Fc receptor-mediated cross-linking of TcR-bound anti-CD3 mAb results in T cell activation marker expression, and proliferation (Weiss, 1986). Similarly, *in vivo* administration of OKT3 results in both T cell activation and suppression of immune responses (Ellenhorn, 1990; Chatenoud, 1990).

OKT3 has been used clinically for over a decade in the treatment of steroid resistant graft rejection (Cosimi *et al.*, 1985; Ortho Multicenter Transplant Study Group, 1985; Thistlewaite *et al.*, 1987). However, use of this antibody has been hampered by a toxic "first

dose reaction syndrome" shown to be related to initial T cell activation events and ensuing release of cytokines prior to the suppression of T cell responses (Thistlewaite *et al.*, 1988; Ferran *et al.*, 1990; Alegre *et al.*, 1990b; Alegre *et al.*, 1990a). Repeated daily administration of OKT3 results in profound immunosuppression, and provides effective treatment of rejection following renal transplantation (Thistlethwaite, 1984). Others have demonstrated that the mitogenic activity of OKT3 and other anti-CD3 mAbs depends upon extensive TCR/CD3 cross-linking via binding to FcR positive cells (Kan *et al.*, 1986).

Reported side effects of OKT3 therapy include flu-like symptoms, respiratory distress, neurological symptoms, and acute tubular necrosis that may follow the first, and sometimes the second, injection of the mAb (Abramowicz, 1989; Chatenoud, 1989; Toussaint, 1989; Thistlethwaite, 1988; Goldman, 1990). It has been shown that the activating properties of OKT3 result from TcR cross-linking mediated by the mAb bound to T cells (via its $F(ab')_2$ portion) and to FctR-bearing cells via its Fc portion) (Palacios, 1985; Ceuppens, 1985; Kan, 1986). Thus, before achieving immunosuppression, OKT3 triggers activation of mAb-bound T cells and FctR-bearing cells, resulting in a massive systemic release of cytokines responsible for the acute toxicity of the mAb (Abramowicz, 1989; Chatenoud, 1989). Data obtained using experimental models in chimpanzees and mice have suggested that preventing or neutralizing the cellular activation induced by anti-CD3 mAbs reduces the toxicity of these agents (Parleviet, 1990; Rao, 1991; Alegre, *Eur. J. Immunol.*, 1990a; Alegre, *Transplant Proc.*, 1990b; Alegre, *Transplantation*, 1991a; Alegre, *J. Immun.*, 1991b; Ferran, *Transplantation*, 1990). In addition, previous results reported in mice using $F(ab')_2$ fragments of 145-2C11 (a hamster anti-mouse CD3 that shares many properties with OKTS3) have suggested that, in the absence of FctR binding and cellular activation, anti-CD3 mAbs retain at least some immunosuppressive properties *in vivo* (Hirsch, *Transplant Proc.*, 1991a; Hirsch, *J. Immunol.*, 1991b).

Therefore, recent efforts have been devoted to developing Fc receptor non-binding forms of anti-CD3 by altering binding to the Fc receptor. As a model system, an anti-murine CD3 mAb, 145-2C11, was genetically altered to eliminate FcR binding. Its variable region gene was fused to a murine IgG3 Fc region, a mouse isotype with low affinity for murine FcR

(U.S. Patent Application Serial Number 08/557,050 and allowed U.S. Patent Application serial No. 08/070,116; each document is specifically incorporated by reference in its entirety). This chimeric anti-CD3-IgG3 antibody has been shown to be Fc receptor non-binding *in vitro*, and did not result in the serum cytokine elevation observed with the whole 145-2C11 mAb *in vivo* (Alegre *et al.*, 1995). However, the administration of Fc receptor non-binding anti-CD3 mAbs was equally effective in prolonging graft survival as the parental 145-2C11 antibody (Alegre *et al.*, 1995). As similar non-FcR binding mAbs derived from OKT3 are being tested clinically, it is important to gain further understanding of the mechanism(s) by which these Fc receptor non-binding mAbs suppress T cell responses.

A great need exists for non-activating forms of anti-human CD3 mAbs for use as immunosuppressive agents that could be administered to recipients undergoing acute transplant rejection, to render activated T cells unresponsive. However, evidence exists that suggests that FcR-nonbinding anti-CD3 mAbs do not suppress all activated Th subsets. In a murine collagen arthritis model, the resolution of disease by FcR-nonbinding anti-CD3 $F(ab')_2$ fragments correlated with suppressed IL-2 and IFN-γ production, and preserved IL-4 production (Hughes *et al.*, 1994). Thus, selective regulation of Th subsets may contribute to the *in vivo* efficacy of FcR-nonbinding anti-CD3 mAbs.

SUMMARY OF THE INVENTION

It is a goal of the present invention to provide the methods of using nonactivating forms of anti-CD3 mAbs and methods of improving the efficacy of these antibodies in a variety of disorders.

Thus, in a primary aspect, the present invention relates to methods of modulating the immune system of a mammal. These methods involve the administration of an immunomodulatory compound to the mammal. In preferred embodiments, the immunomodulatory compound is one that (i) selectively that selectively induces ξ chain tyrosine phosphorylation of a p21 form of ξ of the TCR complex, but does not induce the highly phosphorylated p23 form of ξ, and (ii) triggers ZAP-70 association, but does not

induce tryrosine phosphorylation of associated ZAP-70 tyrosine kinase. Such immunomodulatory compounds can selectively inactivate Th1 and/or IL-2 producing T-cells, while promoting Th2 type T cells. The immunomodulatory compound is combined in a pharmaceutically acceptable vehicle and administered to the mammal in amounts effective to modulate an immune system.

Immunomodulation obtained by the methods of the present invention has many uses. For example, it may be useful when a mammal is receiving a hematopoietic tissue transplant. In other cases the mammal may have a disease such as an autoimmune disease, an infection cancer or other malignancy or immunodeficiency. In many cases, the mammal is a human. By immunomodulation the present invention refers to any scenario that alters the immune system by suppressing or enhancing the immune system. Thus immunosuppression and immunostimulation are subsets of immunomodulation. For additional disclosure on immunomodulation the skilled artisan is referred to US Patent Application Serial Numbers 07/429,729 filed 27 October, 1989; 08/286,805 filed August 5, 1994; 08/459,486, filed June 2, 1995; 08/458,122, filed June 2, 1995; 08/458,462 filed June 2, 1995 and all predecessors to these applications (the entire text of each specifically incorporated herein by reference).

The immunomodulatory compounds employed in the present invention may be of any form that exhibits the desired characteristics. For example, the compound is selected for immunomodulatory activity from a small peptide library, a peptidemimectic that mimic the binding of antibodies exemplified herein or one of these exemplified antibodies or fragments thereof. In some preferred embodiments, the immunomodulatory compound is a monoclonal antibody, and in some particularly preferred embodiments, the monoclonal antibody is a Fc receptor non-binding anti-CD3 monoclonal antibody.

Some aspects of the invention make use of Fc receptor non-binding anti-CD3 monoclonal antibodies that comprise a complementary determining region of the murine anti-CD3 monoclonal antibody OKT3, a human IgG variable framework, and a human IgG constant region, wherein the constant region comprises a point-mutation to render the monoclonal antibody less mitogenic. For example, such antibodies may comprise a mutation

to an alanine at position 234 or a point-mutation to alanine at position 235. In some preferred embodiments, the antibody will comprise a double point-mutation to alanine at position 234 and alanine at position 235. The variable framework and constant region of the Fc receptor non-binding anti-CD3 monoclonal antibody may be selected from any of the many known to those of skill in the art. However, in some presently preferred embodiments, they are of either a human IgG4 or a human IgG1. When a human IgG4 variable framework and constant region are selected, some preferred embodiments comprise a mutation from a phenylalanine to an alanine at position 234 and/or a mutation from a leucine to an alanine at position 235. In some cases, the variable framework and constant region are of a human IgG1 and comprise a mutation from a leucine to an alanine at position 234 and/or a mutation from a leucine to an alanine at position 235. In some embodiments, the monoclonal antibody is directed against non-polymorphic TcR-associated CD3 chains, γ, δ, ε or λ.

The immunomodulatory compound is administered in an amount effective to modulate an immune system. Those of skill in the art will be able to employ methods of determining appropriate dosages know to those of skill and the teachings of this specification to determine appropriate dosage time-courses and amounts. It is anticipated the immunomodulatory compounds will be given in amounts ranging from 1 μg/kg to 20,000 μg/kg. Preferred ranges of compounds will be from 10 μg/kg to 2,000 μg/kg. More preferably, the compounds will be administered in a range of from 10 μg/kg to 1,000 μg/kg, with 100 μg/kg to 400 μg/kg being considered particularly advantageous. The immunomodulatory compound may administered as a bolus or as a series of boluses. Such boluses may be delivered over a staggered time course with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 15, 17, 20, or more days between successive bolus administrations. For additional disclosure on immunostimulation and immunosuppression the skilled artisan is referred to U.S. Patent Application Serial Numbers 07/429,729 filed 27 October, 1989; 08/286,805 filed August 5, 1994; 08/459,486, filed June 2, 1995; 08/458,122, filed June 2, 1995; 08/458,462 filed June 2, 1995 (the entire text of each specifically incorporated herein by reference).

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed

description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1A and FIG. 1B. Sequences of humanized OKT3 variable regions. FIGs. 1A and 1B show the alignments of the OKT3 light chain (FIG. 1A) (SEQ ID NO: 6) and the heavy chain (FIG. 1B) (SEQ ID NO: 10) variable domain amino acid sequence (row 1), the variable domain sequence from the human antibodies chosen as acceptor framework (row 2), and the humanized OKT3 variable domain sequences (row's 3-5) (SEQ ID NOS: 8, 9, 12, 13 AND 14). The CDR choices are singly underlined. Rows 3-5 show only differences from the human acceptor sequence, with the non-CDR differences shown double underlined. Dashes indicate gaps introduced in the sequences to maximize the alignment. Numbering is as Kabat *et al.*, (1987).

FIG. 2A-2K. Amino acid and nucleotide sequence of murine OKT3.

FIG. 3A and FIG. 3B. Relative Affinity Determination. Competition of OKT3 and humanized OKT3 antibodies for antigen against FITC-mOKT3. Increasing concentrations of unlabelled competitor antibody were added to a subsaturating concentration of FITC-mOKT3 tracer antibody, and were incubated with human PBMC for 1 hour at 4° C. Cells were washed and fixed, and the amount of bound and free FITC-mOKT3 was calculated. The affinities of the antibodies were each calculated according to the formula [X] - [mOKTK3] = $(1/K_x)$- $(1/K_a)$, where K_a is the affinity of mOKT3, and ==Kx is the affinity of the competitor

X. [ ] indicates the concentration of competitor at which bound/free tracer binding is $R_o/2$ and R_o is maximal tracer binding (Rao, 1992). FIG. 3A and FIG. 3B show results from separate experiments. solid squares: Orthomune @ OKT3; open circles: cOKT3(γ4); closed triangles: gPLT3-1(γ4); closed circles: gOKT3-5(γ4); open squares: gOKT3-7(γ4); open triangles: mOKT4A.

FIG. 4. Proliferation Assay. Proliferation of human PBMC to anti-CD3 antibody produced by COS cell transfection. PBMC were incubated for 68 hours in the presence of increasing amounts of anti-CD3 antibody, then pulsed with ^{3}H-thymidine for an additional 4 h, and the incorporation of ^{3}H-thymidine quantitated. closed squares: Orthomune@ OKT3; open squares: gOKT3-7(γ4); open triangles: mOKT4A.

FIG. 5. OKT3 displacement assay. Serial dilutions of the "humanized" mAbs were used to competitively inhibit the binding of labeled OKT3 to the CD3 complex, as described in materials and methods. Values are expressed as a percent of the maximal fluorescence (arbitrary units attributed by the flow cytometer) achieved by binding of the labeled OKT3 alone. The symbols correspond to the following Abs: open circles, gOKT3-6 mAb; closed triangles, gOKT3-5 mAb; open squares, Leu-234 mAb; closed circles, Glu-235 mAb.

FIG. 6. N-terminal of CH_2 domain.

FIG. 7. Mitogenicity induced by murine and "humanized" anti-CD3 mAbs. PBMC were incubated for 72 hours with serial dilutions of the mAbs before the addition of 1μCi/well of H^3 Thymidine. Proliferation is depicted as the mean counts per minute (CPM) of triplicates (SEM<10%). These data are representative of the proliferation obtained with PBMC with 3 different donors. The symbols correspond to the following Abs: open triangles, OKT3; closed triangles, gOKT3-5 mAb; closed circles, Glu-235 mAb.

FIG. 8A and FIG. 8B. Expression of markers of activation on the surface of T cells after stimulation with murine and "humanized" OKT3 mAbs. T cell expression of Leu 23 and IL-2 receptor was determined after culture of PBMC for 12 or 36 hours respectively, in

the presence of varying concentrations of the anti-CD3 mAbs. The cells were stained with FITC-coupled anti-Leu 23 or anti-IL-2 receptor Abs and the fraction of T cells (CD2 or CD5-positive cells, counterstained by PE-coupled Abs) expressing the markers of activation were determined by FCM. The symbols correspond to the following Abs: open triangles, OKT3; closed triangles, gOKT3-5 mAb; closed circles, Glu-235 mAb.

FIG. 9. Release of TNF induced by murine and "humanized" OKT3 mAbs. PBMC were cultured with serial dilutions of the different Abs for 24 hours. The concentration of TNF-α was determined by ELISA, using a commercial kit. Values are expressed as the mean of triplicates (SEM<10%). The symbols correspond to the following Abs: open triangles, OKT3; closed triangles, gOKT3-5 mAb; closed circles, Glu-235 mAb.

FIG. 10A, FIG. 10B and FIG. 10C. Modulation and coating of the TCR achieved by the anti-CD3 mAbs. PBMC were incubated for 12 hours with various amounts of the anti-CD3 mAbs. Coating and modulation of the TCR complex was quantitated by FCM as explained in materials and methods. T cells were counterstained with PE-coupled anti-CD5 Ab. The bottom black boxes correspond to the total percentage of CD3 complexes that are modulated, the middle grey boxes to the percentage of CD3 complexes coated by the anti-CD3 mAbs and the upper white dotted boxes to the percentage of CD3 complexes uncoated on the surface of T lymphocytes.

FIG. 11. Inhibition of T cell cytotoxic activity by "humanized" OKT3 mAbs. HLA A2-specific effector CTLs were generated by secondary mixed lymphocyte culture. Lysis of an A2-expressing LCL target was quantitated by a ^{51}Cr-release assay. Values are expressed as percent of maximum specific lysis. (Maximum specific lysis was determined to be 60% of the maximum lysis observed with 0.1 M HCL). Results represent the mean of triplicates (SEM<10%). The symbols correspond to the following Abs: open circles, gOKT3-6 mAb; open triangles; OKT3; closed triangles, gOKT3-5 mAb; closed circles, Glu-235 mAb.

FIG. 12A and FIG. 12B. Variations of mean fluorescence of CD4 and CD8 surface markers induced by anti-CD3 mAbs.

FIG. 13. CD4 binding to RES-KW3 cells.

FIG. 14. CD4 binding on ELISA plates.

FIG. 15. T cell proliferation to "humanized" mAbs. ^{3}H-thymidine incorporation by PBMC induced by soluble anti-CD3 mAbs was examined. Human PBMCs were incubated with serial log dilutions of soluble OKT3 (closed circles), 209-IgG4 (closed squares), 209-IgG1 (closed triangles) or Ala-Ala-IgG4 (closed circles) mAbs for 72 hours, pulsed with ^{3}H-thymidine for an additional 4 hours, and quantified by using scintillation counting. All data is expressed as mean counts per minute of triplicate samples.

FIG. 16. Serum levels of anti-CD3 mAbs. Hu-SPL-SCID mice received OKT3, 209-IgG1 or Ala-Ala-IgG4 (100 μg in 1 ml PBS ip). The animals were bled 1, 2 and 8 days after the injection. Serum levels of anti-CD3 were measured by FCM as described in materials and methods. Results are expressed as Mean ± SEM of 5 animals per group.

FIG. 17. Ala-Ala-IgG4 does not induce upregulation of CD69. Hu-SPL-SCID mice were treated with PBS (1 ml) or OKT3, 209-IgG1 or Ala-Ala-IgG4 (100 μg in 1 ml PBS ip). Spleens were harvested 24h after the injection, prepared into single cell suspensions and analyzed by FCM. The mean fluorescence obtained with anti- human CD69 on $CD4^{+}$ and $CD8^{+}$ human T cells of PBS-treated mice was used as baseline. Results are expressed as the percent increase from that baseline (Mean ± SEM of 5 animals per group) and are representative of 4 independent experiments.

FIG. 18. Production of human IL-2 after injection of anti-CD3 mAbs. Hu-SPL-SCID mice received PBS (1 ml) or 145-2C11, OKT3, 209-IgG1 or Ala-Ala-IgG4 (100 μg in 1 ml PBS ip). Mice were bled 2h after the injection, and sera were analyzed for human IL-2 levels, using a bioassay, as described in materials and methods. Results are displayed as the Mean ± SEM of 4 mice/group, and are representative of 2 independent experiments.

FIG. 19. Prolongation of human allograft survival by anti-CD3 mAbs. SCID (4 mice) and hu-SPL- SCID mice (29 mice) were grafted with allogeneic human foreskin. Hu-SPL-SCID mice were treated with PBS (1 ml/d for 14 days, 4 mice), 145-2C11 (4 mice), OKT3 (8 mice), 209-IgG1 (6 mice) or Ala-Ala-IgG4 (5 mice). mAbs were administered ip at 50 μg/day for 5 days followed by 10 μg/day for 10 days. Results are representative of 3 independent experiments. A two-tailed FISHER EXACT test was used to compare the various groups in the 3 skin graft experiments performed. No difference in efficacy was found between the different Abs as the best results were achieved by different Abs in each experiment (OKT3 vs. 209-IgG: p=0.12; OKT3 vs Ala-Ala-IgG: p=1.0; 209-IgG vs. Ala-Ala-IgG: p=0.23).

FIG. 20A and FIG. 20B. Non-FcR binding anti-CD3 induces proliferation only in the presence of cross-linking anti-Ig antibody. Whole spleen (FIG. 20A), or PGL10 clone cells (FIG. 20A), were cultured with an anti-CD3-IgG3 chimeric antibody and a secondary rabbit anti-mouse IgG3 Ab mAb for 48 hrs. Results are expressed as the mean of triplicate determinations and are representative of four independent studies.

FIG. 21A, FIG. 21B, FIG. 21C and FIG. 21D. T cell clones, but not lymph node T cells, are hyporesponsive after exposure to Fc receptor non-binding anti-CD3. (FIG. 21A) DO 11.10 lymph node cells or pGL10 cells were incubated with either media alone or anti-CD3-IgG3 in the presence of irradiated T-depleted spleen cells for 24 hrs, washed, and rested for 72 hrs. The T cells were restimulated with mitogenic anti-CD3 (145-2C11) and fresh APC. (FIG. 21B) AE.7 clone cells were incubated with or without Fc receptor non-binding anti-CD3, washed and rested as above, and restimulated with the antigen PCC plus fresh APC. (FIG. 21C) pGL10 cells were incubated with or without Fc receptor non-binding anti-CD3. After the 72 hour rest, the pGL10 were restimulated with immobilized anti-CD3 plus anti-CD28. Culture supernatants were analyzed by IL-2 ELISA. (FIG. 21D) pGL10 cells were incubated with Fc receptor non-binding anti-CD3 in the presence of Cyclosporine A, splenic APC and anti-CD28 as indicated. 72 hours after the primary culture, cells were

restimulated with OVA antigen and APC. (FIG. 21A), (FIG. 21C), and (FIG. 21D) are representative of two separate studies, and (FIG. 21B) is representative of four studies.

FIG. 22. Partial tyrosine phosphorylation of TCR components by Fc receptor non-binding anti-CD3. Densitometry was performed on results from four independent studies to quantitate the relative amounts of p21 and p23 phosphorylated ζ. In each studies, the p23/p21 ratios for cross-linked anti-CD3 (hatched bars) and non-cross-linked anti-CD3 (open bars) are represented.

FIG. 23. Impaired PLCγ-1 activation and Ca^{2+} flux in the absence of anti-CD3 cross-linking. T cell clones were loaded with the calcium sensitive dye indo-1, stimulated with anti-CD3-IgG3 (left), or anti-CD3-IgG3 followed by rabbit anti-IgG3 (right). Cells were analyzed on a FACStar plus for calcium flux. The rise in relative intracellular calcium concentration is indicated by an increase in the 405/495 nm emission ratio. Data is representative of two separate studies.

FIG. 24A and FIG. 24B. Stimulation of anti-CD3 x anti-CD4 results in increased phosphorylation of proteins associated with the TCR complex and reconstitutes a mitogenic stimulus. Wholes spleen (FIG. 24A) or pGL10 T cells (FIG. 24B) were cultured with serial log dilutions of anti-CD3 Fos (open diamonds) or bispecific anti-CD3 x anti-CD4 (closed diamonds) for 48 hours. Data is representative of three separate experiments.

FIG. 25A and FIG. 25B. Proliferative response to immobilized vs. soluble anti-CD-3. FIG. 25A depicts pGL10(Th1) and FIG. 25B depicts pL104(Th2).

FIG. 26A and FIG. 26B. Non clonal activated T cells produce IL-4 (FIG. 26B) but not IL-2 (FIG. 26A) in response to 2C11-IgG3.

FIG. 27. Th2 clones produce IL-4 in the secondary stimulation.

FIG. 28A and FIG. 28B. FcR non-binding anti-CD3 monoclonal antibodies induce anergy in Th1 but not Th2 clones.

FIG. 29A, FIG. 29B and FIG. 29C. Anti-CD3 IgG3 induces IL-4 production and proliferation in Th2 clones. FIG. 29A and FIG. 29B. pGL10 (Th1; FIG. 29A) or pL104 (Th2; FIG. 29B) T cell clones were cultured in the presence of media, soluble anti-CD3 IgG3 (open squares) or plastic immobilized anti-CD3 (filled squares) for 40 h and then pulsed for 8 h with [3H]TdR. FIG. 29C. Supernatants (40 h) were examined for the presence of IL-4 by ELISA. Results are representative of three independent studies.

FIG. 30A and FIG. 30B. Th0 clones proliferate and produce IL-4 in response to the anti-CD3 IgG3 mAb. FIG. 30A. The Th0 clone 4.5 was cultured in the presence of media, soluble anti-CD3 IgG3, or immobilized anti-CD3 for 40 h, and then pulsed for 8 h. FIG. 30B. A second Th0 clone, 24.5, was stimulated as indicated, and then 40-h supernatants were tested by ELISA for IL-2 (open bars) and IL-4 (hatched bars) production. Results are representative of three separate studies. In individual Th0 studies, similar results were obtained with clone 4.5 and 24.5. * = Below limit of detection (0.2 ng/ml).

FIG. 31A, FIG. 31B and FIG. 31C. Anti-IL-4 mAb, but not anti-IL-2/IL-2R mAbs, block anti-CD3 IgG3-induced proliferation in a Th0 T cell clone. T cells (Th0) were stimulated with 4.5 1 μg/ml of soluble anti-CD3 IgG3 (FIG. 31A) or anti-CD3 in the presence (FIG. 31B) or absence of APC (FIG. 31C). Anti-IL-4 mAb, anti-IL-2/IL-2R, or rat control Ig were added as indicated. Cultures were pulsed with [^{3}H]TdR at 40 h. Results are representative of four independent studies. Similar results were obtained with the Th0 clone 24.5.

FIG. 32A, FIG. 32B and FIG. 32C. Polyclonal activated T cell populations produce IL-4 and proliferate in response to anti-CD3 IgG3. DO 11.10 lymph node cells were activated with OVA peptide, irradiated splenic APC, and IL-2 one to three times *in vitro*. The T cells were then cultured with media, anti-CD3 IgG3, or immobilized anti-CD3 for 40 h, and pulsed with [^{3}H]TdR for 8 h. Proliferation results are representative of four

independent studies (FIG. 32A). Supernatants were harvested at 40 h and analyzed by ELISA for IL-2 (FIG. 32B) and IL-4 (FIG. 32C) production. Similar results were obtained with supernatants harvested at 24 h.

FIG. 33A and FIG. 33B. Soluble anti-CD3 IgG3 induces proliferation in *in vitro*-activated IFN-γKO T cells (FIG. 33A), but not IL-4KO T cells (FIG. 33B). CD8-depleted lymph node cells from IL-4KO or IFN-γKO mice were activated *in vitro* one or two times with anti-CD3 (145-2C11), IL-2, and T-depleted irradiated splenic APC. The activated T cells were then cultured with soluble or immobilized anti-CD3 for 48 h. Results are representative of three separate studies.

FIG. 34A, FIG. 34B and FIG. 34C. Anti-CD3 IgG3 renders Th1 and Th0 clones, but not Th2 clones, unresponsive. pGL10 (Th1; FIG. 34A) or pL104 (Th2; FIG. 34B) clones were cultured with media alone or soluble anti-CD3 IgG3 for 24 h, washed three times, and then rested for 3 days. At this point, the T cell clones were restimulated with 1 μg/ml of OVA Ag and T-depleted irradiated splenic APC for 48 h, and then pulsed for a further 12 to 16 h. Supernatants were harvested at 48 h and examined for the presence of IL-4 by ELISA. Results are representative of two independent studies. FIG. 34C. Th0 clones were cultured with or without anti-CD3 IgG3, and restimulated as in FIG. 34A and FIG. 34B. Three studies were performed using both Th0 clones 24.5 and 4.5 (similar proliferation results were obtained with each). Over multiple studies, IL-4 production by anti-CD3 IgG3-pretreated T cells during the secondary stimulation ranged from 40 to 240% of media pretreated controls.

FIG. 35A, FIG. 35B and FIG. 35C. Anti-CD3 IgG3 treatment of polyclonal populations results in decreased IL-2 production. Bulk T cells, activated in FIG. 32, were cultured with anti-CD3 IgG3 for 24 h, washed, rested, and restimulated with OVA peptide (or OVA Ag) and T-depleted irradiated splenic APC for 48 h. At 48 h, supernatants were collected for analysis by ELISA and cultures were pulsed with [^{3}H]TdR. Cytokine results are representative of seven independent studies. In the restimulation, proliferation results varied from no effect (as shown) up to a 67% decrease after pretreatment with anti-CD3 IgG3.

Proliferation is shown in FIG. 35A, IL-2 production in FIG. 35B and IL-4 production in FIG. 35C.

FIG. 36A and FIG. 36B. Soluble anti-CD3 IgG3 induces hyporesponsiveness in activated IL-4KO (FIG. 36A), but not IFNγKO T cells (FIG. 36B). Cytokine KO T cells were activated as in FIG. 33, cultured with anti-CD3 IgG3 or media for 24 h, and then rested for 72 h. In the restimulation, the T cells were stimulated with anti-CD3 (145-2C11) and T-depleted irradiated splenic APC. Results are representative of two independent studies.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The potent immunosuppressive agent OKT3 is a murine IgG2a mAb directed against the CD3 complex associated with the human TCR (Van Wauwe, 1980). However, the administration of OKT3 to transplant recipients induces the systematic release of several cytokines, including IL-2, IL-6, TNF-α and IFN-γ (Abramowicz, 1989; Chatenoud, 1989). This production of cytokines has been correlated with the adverse side-effects frequently observed after the first injection of OKT3 (Van Wauwe, 1980; Chatenoud, 1989; Thistlethwaite, 1988). The cytokine production also may augment the production of anti-isotopic and anti-idiotypic antibodies occurring in some patients after one or two weeks of treatment. These events then can neutralize OKT3 and preclude subsequent treatments of graft rejection episodes (Thistlethwaite, 1988).

Several pieces of evidence strongly suggest that these side-effects are a consequence of the cross-linking between T lymphocytes and Fc receptor (FcR)-bearing cells through the Fc portion of OKT3, resulting in activation of both cell types (Debets, 1990; Krutman, 1990): 1) anti-CD3 mAbs did not stimulate T cell proliferation *in vitro*, unless the Ab was immobilized to plastic or bound to FCR+ antigen presenting cells included in the culture (van Lier, 1989); 2) the cross-linking of OKT3 through FcRs I and II enhanced proliferation in response to IL-2, *in vitro* (van Lier, 1987a and 1987b); 3) proliferation of murine T cells induced by 145-2C11, a hamster mAb directed against the murine CD3 complex, could be blocked by the anti-FcR Ab, 2.4G2; 4) the injection into mice of $F(ab')_2$ fragments of 145-

2C11 induced significant immunosuppression without triggering full T cell activation (Hirsch, 1990) and was less toxic in mice than the whole mAb (Alegre, 1990a and 1990b) and 5) the administration of an OKT3 IgA switch variant that displayed a reduced FcR-mediated T cell activation as compared with OKT3 IgG2a, resulted in fewer side effects in chimpanzees *in vivo* (Parleviet, 1990).

The activation-related adverse side effects associated with these mAbs have prompted the development of less toxic Fc receptor non-binding anti-CD3 mAb therapies. At present, the functional and biochemical consequences of T cell exposure to Fc receptor non-binding anti-CD3 is unclear.

I. Detailed Description of The Present Invention

In this invention, the inventors have examined the early signaling events triggered by a Fc receptor non-binding anti-CD3 mAb. Like the mitogenic anti-CD3 mAb (OKT3), Fc receptor non-binding anti-CD3 triggers changes in the TCR complex, including ζ chain tyrosine phosphorylation and ZAP-70 association. However, unlike the mitogenic anti-CD3 stimulation, Fc receptor non-binding anti-CD3 was ineffective at inducing the highly phosphorylated form of ζ (p23) and tyrosine phosphorylation of the associated ZAP-70 tyrosine kinase.

This proximal signaling deficiency correlates with minimal PLCγ-1 phosphorylation and failure to mobilize detectable Ca^{2+}. Not only did biochemical signals delivered by Fc receptor non-binding anti-CD3 resemble altered peptide ligand signaling, but exposure of Th1 clones to Fc receptor non-binding anti-CD3 also result in functional anergy. Finally, a bispecific anti-CD3 x anti-CD4 $F(ab)'_2$ reconstitutes early signal transduction events and induces proliferation, suggesting that defective association of lck with the TCR complex may underlie the observed signaling differences between the mitogenic and Fc receptor non-binding anti-CD3.

During the course of an immune response, naive T cells differentiate into Th phenotypes defined by their pattern of cytokine secretion and immunomodulatory properties.

More particularly, Th1 cells secrete TNF-α, IL-2, and IFN-γ, which enhance inflammatory cell-mediated responses, whereas Th2 cells secrete IL-4, IL-5, IL-10, and IL-13, cytokines that suppress inflammatory responses while potentiating humoral immunity (Abbas, 1996). Multiple studies have suggested that the induction and maintenance of tolerance in both transplant and autoimmune diseases is a direct consequence of enhanced Th2 activity at the expense of the Th1 subset (Strom *et al.*, 1996; Nicholson and Kuchroo, 1996). For example, treatments that prolong graft survival, such as CTLA4-Ig and anti-CD4 mAbs, correlate with increased IL-4 and IL-10 production in accepted grafts (Sayegh *et al.*, 1995; Mouram *et al.*, 1995). Thus, any treatment that might "tip the balance" toward a Th2 phenotype would have important therapeutic implications.

The present invention has demonstrates that Th2 clones and polyclonal IL-4-secreting T cell populations proliferated, and were not rendered unresponsive by the FcR-nonbinding anti-CD3 mAbs. Furthermore, polyclonal activated populations exposed to FcR-nonbinding anti-CD3 maintained their ability to produce IL-4, but secreted much less IL-2 in a secondary response. The results suggest FcR-nonbinding anti-CD3 delivers a partial signal that has different functional consequences for Th1 or Th2 populations. The promotion of Th2 cytokine secretion and proliferation, and the concomitant suppression of Th1 responses are likely to account for the ability of FcR-nonbinding anti-CD3 to skew *in vivo* immune responses toward a Th2 phenotype.

Together these observations indicate that administration of Fc receptor non-binding anti-CD3 mAbs, especially in recipients undergoing acute transplant rejection, will likely result in the delivery of a partial T cell signal that selectively renders activated T cells unresponsive. Thus in particular embodiments, it is envisioned that the immune system of a mammal may be modulated by providing a composition that comprises an immunomodulatory compound that selectively induces ξ chain tyrosine phosphorylation of a p21 form of ξ of the TCR complex without induction of the highly phosphorylated p23 form of ξ and triggers ZAP-70 association, but does not induce tryrosine phosphorylation of associated ZAP-70 tyrosine kinase.

II. The Effects of Anti-CD-3 Antibodies

a. Anti-CD3 mediated immunosuppression

The mechanism of immunosuppression by anti-CD3 mAbs is complex. Mitogenic anti-CD3 mAbs, such as OKT3, modulate the TCR, induce apoptosis and induce generalized long term T cell unresponsiveness (Hirsch *et al.*, 1988). Similarly, treatment of mice with the Fc receptor non-binding anti-CD3 results in internalization of the TCR complex and depletion of T cells from the circulation and peripheral lymphoid organs. However, in contrast to the mitogenic antibodies, anti-CD3-IgG3 does not appear to induce global T cell unresponsiveness (Alegre *et al.*, 1995). Thus, the various anti-CD3 mAbs may suppress T cell responses by distinct mechanisms. Treatment with anti-CD3-IgG3 alters expression of several T cell surface molecules; both CD44 and Ly6-C are upregulated following exposure to the chimeric anti-CD3 (Alegre, 1993). Thus the interaction of anti-CD3-IgG3 with T cells is not inert, but may deliver at least a partial TCR signal that contributes to its immunosuppressive activity.

It is thought that TCR signaling results from a cascade of events requiring the recruitment and activation of non-receptor tyrosine kinases. One of the earliest consequences of TCR engagement by mAb or peptide/MHC is the tyrosine phosphorylation of components of the TCR complex (Qian *et al.*, 1993). The ζ chain of the TCR complex contains 3 ITAM motifs ($D/EXXYXXL(X)_{6-8}$ YXXL) that become variably phosphorylated following TCR/CD3 ligation (Weiss and Littman, 1994). It is thought that the activation-induced 21 and 23 kd phosphorylated bands, evident on one dimensional SDS-PAGE, represent differentially phosphorylated forms of ζ (Sloan-Lancaster *et al.*, 1994).

The other CD3 chains-- γ, δ and ε (containing one ITAM each)-- become tyrosine phosphorylated as well (Qian *et al.*, 1993). It has been hypothesized that the src family kinases, lck or fyn, may be responsible for these early phosphorylation events (Weiss and Littman, 1994). Within minutes, additional tyrosine phosphorylated proteins, including the ZAP-70 kinase, associate with the TCR/CD3 complex (Straus and Weiss, 1993; Chan *et al.*, 1991). These proximal events lead to a series of biochemical signals that activate

downstream substrates in the PI-3 kinase, Ras and Phospholipase Cγ-1 (PLCγ-1) pathways, ultimately leading to activation of the T cell (Weiss and Littman, 1994).

Until recently, it was thought that this cascade of events was always fully engaged following exposure to peptide/MHC ligand or mAbs and that different responses to stimuli reflected a quantitative addition of the number of receptors engaged. However, antigenic peptide analogues, designated as altered peptide ligands (APL), have illustrated that the TCR is not an "on-off" switch. Rather, stimulation with APL can result in qualitative differences in the early signals transduced through the TCR. Specifically, stimulation with APL results in a characteristic biochemical pattern involving partial ζ phosphorylation and ZAP-70 association in the absence of phosphorylation, ultimately leading to a lack of Inositol-trisphosphate (IP_3) turnover (Sloan-Lancaster *et al.*, 1994; Sloan-Lancaster *et al.*, 1993; Madrenas *et al.*, 1995). The delivery of such a partial signal effectively shuts down T cell clones, resulting in the induction of unresponsiveness as manifested by an inability of the "anergized" T cell clones to produce IL-2 when re-challenged under optimal conditions. In support of this hypothesis, a recent study has demonstrated that low dose anti-CD3 or non-mitogenic anti-CD3 $F(ab')_2$ fragments induce tolerance in overtly diabetic NOD mice, but do not prevent diabetes if administered before disease onset (Chatenoud *et al.*, 1997). Thus, the mechanism by which these mAbs suppress immune responses may depend upon the selective effects of FcR-nonbinding anti-CD3 on activated T cells.

b. Partial Signaling By Bivalent Anti-CD3 Antibodies

The present invention is based in part on the discovery that bivalent anti-CD3 delivers a partial TCR signal which renders Th1 clones hyporesponsive. This signal consists of phosphorylation of several components of the TCR complex, (bands representing CD3 ε, δ), ZAP-70 association, and partial phosphorylation of TCR ζ; in the absence of cross-linking, there is a relatively greater induction of the phosphorylated p21 ζ as compared to the p23 ζ band species evident in T cell clones.

p21 induction appears to be sufficient for association of the ZAP-70 kinase with the TCR complex, whereas p23 induction and ZAP-70 phosphorylation appear to be interrelated

events. Indeed, the low level of ZAP-70 phosphorylation observed in the non-cross-linked situation correlates with the small amount of p23 ζ that is generated.

In a recent study, Weist *et al.* proposed that the p23 form of ζ observed in thymocytes upon *in vitro* stimulation depends on greater TCR aggregation (Wiest *et al.*, 1996). The inventors' findings are consistent with this hypothesis. Higher orders of TCR aggregation also appear to be required for recruitment of other phosphotyrosine containing molecules to the TCR/CD3 complex in both T cell clones and bulk naive cells. If any of these tyrosine phosphorylated molecules contain SH2 domains, they may require the fully phosphorylated p23 form of ζ for association. Alternatively, the p23 form may be required for "docking" of a kinase which phosphorylates these associated molecules.

The observation that non-cross-linked anti-CD3 induces less ZAP-70 phosphorylation and p23 phospho-ζ bears a striking resemblance to the findings in the altered peptide ligand studies (Sloan-Lancaster *et al.*, 1994; Madrenas *et al.*, 1995). The relative contribution of affinity for MHC or TCR (and thus occupancy) *vs.* TCR aggregation has been unclear in these systems. Recently, Lyons *et al.* showed a correlation between antagonist activity of certain altered peptide ligands and a higher TCR dissociation rate (Lyons *et al.*, 1996). However, this finding does not exclude a role for aggregation in that a shorter dwell time of the TCR may fail to induce the oligomerization required for a fully activating stimulus.

In the present invention, the issue of affinity has been addressed: the same primary antibody was used in both cross-linked and non-cross-linked situations. Thus, intrinsic affinity for TCR was held constant. Since similar signaling deficits were found in non-cross-linked anti-CD3 and altered peptide ligand stimulations, it is possible that the altered peptide ligands may induce their characteristic partial signals because of insufficient TCR aggregation.

c. Lck Recruitment Into The Complex Reconstitutes Signal Transduction And Mitogenicity

There are several ways in which the localization of multiple TCR complexes within a large aggregate could enhance signaling. In the "kinetic proofreading model" proposed by McKeithan (1995), TCR signal transduction was modeled as a reversible multi-step pathway containing sequential phosphorylation events. In this paradigm, aggregation of TCRs might enhance propagation of the signal by favoring phosphorylation over dephosphorylation (McKeithan, 1995).

On a more mechanistic level, aggregation may aid in recruiting key signaling molecules; recruitment of molecules may be further stabilized if there are multiple potential contact points (catalytic sites, SH2 domains, or other recognition motifs) between components of the TCR complex that are in close proximity. For example, if lck binds one phosphorylated ZAP-70 through its SH2 domain, the lck would be in a prime position to phosphorylate a neighboring ZAP-70 molecule in the TCR aggregate. In the non cross-linked situation, lck might migrate away before phosphorylating more ZAP-70 molecules. Thus, aggregated TCR signal transduction may result in amplification of these signals, since one kinase may act on multiple substrates. This capacity for amplification would mean that proximal differences should become magnified as the signal is propagated. As seen in the present study, a relative reduction in ZAP-70 phosphorylation leads to a more dramatic deficiency in PLCγ-1 phosphorylation and undetectable Ca^{++} flux.

The redistribution of TCRs to one pole, within minutes upon addition of secondary cross-linker to anti-CD3, is likely to reflect changes in the underlying cytoskeleton. Others have shown that TCR engagement can lead to redistribution of cytoskeletal elements such as talin, vinculin, and actin (Selliah *et al.*, 1996). The cross-linking Ab might be providing sufficient TCR aggregation to trigger a threshold signal for cytoskeletal mobilization. Studies by Valetutti *et al.* (1995) have suggested that the cytoskeleton also plays an active role in sustaining a TCR signal since the addition of agents which disrupt the actin cytoskeleton (*e.g.* Cytocholasin D) can block the rise in intracellular Ca^{2+} (Valitutti *et al.*, 1995). The

cross-linked anti-CD3 system may be useful for dissecting the role of the cytoskeleton in proximal signaling events.

The inventors' observations suggest that efficient recruitment of lck may be the pivotal event accomplished by aggregation. Lck has been shown to be important for proximal signaling in that absence of lck almost completely abrogates tyrosine phosphorylation events (Straus and Weiss, 1992; van Oers *et al.*, 1996). It is well established that coaggregating anti-CD3 and anti-CD4 antibodies or using anti-CD3/anti-CD4 heteroconjugate mAbs can result in enhanced tyrosine phosphorylation and calcium mobilization (Ledbetter *et al.*, 1988).

Recently, it was shown that in circumstances in which lck is limiting, as in double positive thymocytes, ZAP-70 phosphorylation requires coaggregation of TCR and CD4 (Wiest *et al.*, 1996). Furthermore, blockading CD4 (and presumably its associated lck molecules) with anti-CD4 mAbs can convert a partial agonist signal into an antagonist signal with its associated characteristic signaling deficits (Mannie *et al.*, 1995). Thus impaired CD4 recruitment has been proposed as a mechanism for altered peptide/antagonist peptide signaling.

The pivotal nature of lck recruitment is underscored by the inventors' finding that secondary antibody induced aggregation can be dispensed with, if lck is recruited by bringing CD4 into the complex artificially. Even in the absence of exogenous cross-linking, stimulation with a bivalent anti-CD3 × anti-CD4 reagent reconstituted both the early signaling events of ZAP-70 phosphorylation and association of other phosphorylated proteins with the complex. In turn, these early events lead ultimately to a mitogenic stimulus.

The partial signals delivered by Fc receptor non-binding anti-CD3 correlated with the induction of functional anergy as defined by an inability to proliferate due to poor IL-2 production. The striking similarity between the signals delivered by altered peptide ligands and Fc receptor non-binding anti-CD3 are perhaps indicative of a common mechanism of anergy induction. How these partial signals translate into an "off" signal which shuts down

T cell clonal responsiveness has yet to be determined. In the classical model of anergy, involving a complete signal one (through the TCR) in the absence of signal two (costimulation), induction of unresponsiveness depends upon a successful calcium signal which can be blocked by CsA (Jenkins *et al.*, 1990; Schwartz *et al.*, 1989; Jenkins and Schwartz, 1987). Similarly, CsA has been shown to block anergy induction by altered peptide ligands (Sloan-Lancaster *et al.*, 1993). In fact, an altered peptide ligand triggered calcium signal has been recently demonstrated using an exquisitely sensitive system (Sloan-Lancaster *et al.*, 1996). The ability of CsA to block Fc receptor non-binding anti-CD3 induced functional anergy suggests that a calcium signal may be important in this process. It is possible that the lack of detectable calcium flux by Fc receptor non-binding anti-CD3 reflects insufficient sensitivity. In contrast to the classical models of anergy, the presence of competent APC or anti-CD28 antibodies did not rescue T cell clones from Fc receptor non-binding anti-CD3 induced unresponsiveness.

d. Fc Receptor Non-Binding Anti-CD3 Has Differential Effects On Activated T Cell Subsets

An important observation was the finding that culture with the Fc receptor non-binding anti-CD3 suppresses IL-2 production in clones, but it did not appear to significantly impair the responsiveness of bulk T cells. The inventors' results suggest similar defects in signaling between naive cells and clones in terms of ZAP-70 phosphorylation and TCR/CD3 complex associated phosphorylated molecules, as well as the downstream events of PLCγ-1 phosphorylation and TCR capping. It is possible that naive cells and clones differ in the way they respond to Fc receptor non-binding anti-CD3 mAbs, either in the triggering of other biochemical signals, or the integration of downstream nuclear signals.

The mitogenic forms of anti-CD3 currently in use severely suppress global T cell responses. The present invention shows that Fc receptor non-binding anti-CD3 selectively induces unresponsiveness in activated T cell subsets. These findings bear important implications for transplant therapy in that it would be beneficial to be able to suppress the alloreactive T cells which mediate graft rejection while maintaining the responsiveness of other T cells.

To gain a better understanding of the consequences of FcR-nonbinding anti-CD3 treatment for Th responses, an *in vitro* analysis of the mAb's effect on different populations of activated $CD4^+$ T cells was undertaken. In contrast to what had been observed in Th1 clones, Th2 clones and polyclonal IL-4-secreting T cell populations proliferated, and were not rendered unresponsive by the FcR-nonbinding anti-CD3 mAbs. Moreover, polyclonal activated populations exposed to FcR-nonbinding anti-CD3 maintained their ability to produce IL-4, but secreted much less IL-2 in a secondary response. Examination of the proximal signals induced by FcR-nonbinding anti-CD3 mAb in Th1 and Th2 cells revealed qualitatively similar deficiencies in ζ, ZAP-70, and MAP kinase phosphorylation. The reduced proximal signals were sufficient to drive NF-ATc translocation in both Th subsets. Together, these results suggest FcR-nonbinding anti-CD3 delivers a partial signal that has different functional consequences for Th1 or Th2 populations. The promotion of Th2 cytokine secretion and proliferation, and the concomitant suppression of Th1 responses are likely to account for the ability of FcR-nonbinding anti-CD3 to skew *in vivo* immune responses toward a Th2 phenotype.

Thus, the results presented herein have specific implications for the use of FcR-nonbinding Abs in the clinical setting. During an *in vivo* immune response, such as graft rejection, T cells differentiate into both Th1 and Th2 phenotypes. Besides the direct pro-Th2 effect of FcR-nonbinding anti-CD3 mAbs on activated cells, the development of a Th2 response could be magnified through recruitment of uncommitted cells. Cytokines, such as IL-4, promote selective Th development (Abbas *et al.*, 1996). Thus, anti-CD3 IgG3-induced modification of the cytokine milieu could alter Th differentiation of naive T cells responding to Ag. The ability of anti-CD3 IgG3 to suppress Th1 responses while promoting Th2 responses *in vitro* suggests a mechanism that may explain the efficacy of these mAbs in prolonging graft survival in the absence of global anergy induction. Both the low toxicity of FcR-nonbinding anti-CD3 mAbs and their potential for Th2 cytokine deviation show that these Abs will be effective in suppressing Th1-mediated autoimmune diseases.

III. The Immune System.

The immune system of both humans and animals include two principal classes of lymphocytes: the thymus derived cells (T cells), and the bone marrow derived cells (B cells). Mature T cells emerge from the thymus and circulate between the tissues, lymphatics, and the bloodstream. T cells exhibit immunological specificity and are directly involved in cell-mediated immune responses (such as graft rejection). T cells act against or in response to a variety of foreign structures (antigens). In many instances these foreign antigens are expressed on host cells as a result of infection. However, foreign antigens can also come from the host having been altered by neoplasia or infection. Although T cells do not themselves secrete antibodies, they are usually required for antibody secretion by the second class of lymphocytes, B cells.

a. T cells.

There are various subsets of T cells , which are generally defined by antigenic determinants found on their cell surfaces, as well as functional activity and foreign antigen recognition. Some subsets of T cells, such as $CD8^+$ cells, are killer/suppressor cells that play a regulating function in the immune system, while others, such as $CD4^+$ cells, serve to promote inflammatory and humoral responses. (CD refers to cell differentiation cluster; the accompanying numbers are provided in accordance with terminology set forth by the International Workshops on Leukocyte Differentiation, *Immunology Today*, 10:254 (1989). A general reference for all aspects of the immune system may be found in Klein, J. *Immunology: The Science of Self-Nonself Discrimination*, Wiley & Sons, N.Y. (1982).

i. T cell activation.

Human peripheral T lymphocytes can be stimulated to undergo mitosis by a variety of agents including foreign antigens, monoclonal antibodies and lectins such as phytohemagglutinin and concanavalin A. Although activation presumably occurs by binding of the mitogens to specific sites on cell membranes, the nature of these receptors, and their mechanism of activation, is not completely elucidated. Induction of proliferation is only one indication of T cell activation. Other indications of activation, defined as alterations in the

basal or resting state of the cell, include increased lymphokine production and cytotoxic cell activity.

T cell activation is an unexpectedly complex phenomenon that depends on the participation of a variety of cell surface molecules expressed on the responding T cell population (Leo, 1987; Weiss, 1984). For example, the antigen-specific T cell receptor (TcR) is composed of a disulfide-linked heterodimer, containing two clonally distributed, integral membrane glycoprotein chains, α and β, or γ and δ, non-covalently associated with a complex of low molecular weight invariant proteins, commonly designated as CD3 (the older terminology is T3) Leo, 1987).

The TcR α and β chains determine antigen specificities (Saito, 1987). The CD3 structures are thought to represent accessory molecules that may be the transducing elements of activation signals initiated upon binding of the TcR $\alpha\beta$ to its ligand. There are both constant regions of the glycoprotein chains of TcR, and variable regions (polymorphisms). Polymorphic TcR variable regions define subsets of T cells, with distinct specificities. Unlike antibodies which recognize soluble whole foreign proteins as antigen, the TcR complex interacts with small peptidic antigen presented in the context of major histocompatibility complex (MHC) proteins. The MHC proteins represent another highly polymorphic set of molecules randomly dispersed throughout the species. Thus, activation usually requires the tripartite interaction of the TcR and foreign peptidic antigen bound to the major MHC proteins.

With regard to foreign antigen recognition by T cells the number of peptides that are present in sufficient quantities to bind both the polymorphic MHC and be recognized by a given T cell receptor, thus inducing immune response as a practical mechanism, is small. One of the major problems in clinical immunology is that the polymorphic antigens of the MHC impose severe restrictions on triggering an immune response. Another problem is that doses of an invading antigen may be too low to trigger an immune response. By the time the antigenic level rises, it may be too late for the immune system to save the organism.

The tremendous heterogeneity of the MHC proteins among individuals remains the most serious limiting factor in the clinical application of allograft transplantation. The ability to find two individuals whose MHC is identical is extremely rare. Thus, T cells from transplant recipients invariably recognize the donor organ as foreign. Attempts to suppress the alloreactivity by drugs or irradiation has resulted in severe side effects that limit their usefulness. Therefore, more recent experimental and clinical studies have involved the use of antibody therapy to alter immune function *in vivo*. The first successful attempt to develop a more selective immunosuppressive therapy in many was the use of polyclonal heterologous anti-lymphocyte antisera (ATG) (Starzl, 1967).

ii. Antibody structure.

Antibodies comprise a large family of glycoproteins with common structural features. An antibody comprises of four polypeptides that form a three dimensional structure which resembles the letter Y. Typically, an antibody comprises of two different polypeptides, the heavy chain and the light chain.

An antibody molecule typically consists of three functional domains: the Fc, Fab, and antigen binding site. The Fc domain is located at the base of the Y. The arms of the Y comprise the Fab domains. The antigen binding site is located at the end of each arm of the Y.

There are five different types of heavy chain polypeptides which types are designated α, δ, ε, γ, and μ. There are two different types of light chain polypeptides designated k and \. An antibody typically contains only one type of heavy chain and only one type of light chain, although any light chain can associate with any heavy chain.

Antibody molecules are categorized into five classes, IgG, IgM, IgA, IgE and IgD. An antibody molecule comprises one or more Y-units, each Y comprising two heavy chains and two light chains. For example IgG consists of a single Y-unit and has the formula $_2k_2$ or $_2l_2$. IgM comprises of 5 Y-like units.

The amino terminal of each heavy light chain polypeptide is known as the constant (C) region. The carboxyl terminal of each heavy and light chain polypeptide is known as the variable (V) region. Within the variable regions of the chains are Hypervariable regions known as the complementarity determining region (CDR). The variable regions of one heavy chain and one light chain associate to form an antigen binding site. Each heavy chain and each light chain includes three CDRs. The six CDRs of an antigen binding site define the amino acid residues that form the actual binding site for the antigen. The variability of the CDRs account for the diversity of antigen recognition.

b. Immune Response.

The principal function of the immune system is to protect animals from infectious organisms and from their toxic products. This system has evolved a powerful range of mechanisms to locate foreign cells, viruses, or macromolecules; to neutralize these invaders; and to eliminate them from the body. This surveillance is performed by proteins and cells that circulate throughout the body. Many different mechanisms constitute this surveillance, and they can be divided into two broad categories -- nonadaptive and adaptive immunity.

Adaptive immunity is directed against specific molecules and is enhanced by re-exposure. Adaptive immunity is mediated by lymphocytes, which synthesize cell-surface receptors or secrete proteins that bind specifically to foreign molecules. These secreted proteins are known as antibodies. Any molecule that can bind to an antibody is known as an antigen. When a molecule is used to induce an adaptive response it is called an immunogen. The terms "antigen" and "immunogen" are used to describe different properties of a molecule. Immunogenicity is not an intrinsic property of any molecule, but is defined only by its ability to induce an adaptive response. Antigenicity also is not an intrinsic property of a molecule, but is defined by its ability to be bound by an antibody.

The term "immunoglobulin" is often used interchangeably with "antibody." Formally, an antibody is a molecule that binds to a known antigen, while immunoglobulin refers to this group of proteins irrespective of whether or not their binding target is known. This distinction is trivial and the terms are used interchangeably.

Many types of lymphocytes with different functions have been identified. Most of the cellular functions of the immune system can be described by grouping lymphocytes into three basic types -- B cells, cytotoxic T cells, and helper T cells. All three carry cell-surface receptors that can bind antigens. B cells secrete antibodies, and carry a modified form of the same antibody on their surface, where it acts as a receptor for antigens. Cytotoxic T cells lyse foreign or infected cells, and they bind to these target cells through their surface antigen receptor, known as the T-cell receptor. Helper T cells play a key regulatory role in controlling the response of B cells and cytotoxic T cells, and they also have T-cell receptors on their surface.

The immune system is challenged constantly by an enormous number of antigens. One of the key features of the immune system is that it can synthesize a vast repertoire of antibodies and cell-surface receptors, each with a different antigen binding site. The binding of the antibodies and T-cell receptors to foreign molecules provides the molecular basis for the specificity of the immune response.

The specificity of the immune response is controlled by a simple mechanism -- one cell recognizes one antigen because all of the antigen receptors on a single lymphocyte are identical. This is true for both T and B lymphocytes, even though the types of responses made by these cells are different.

All antigen receptors are glycoproteins found on the surface of mature lymphocytes. Somatic recombination, mutation, and other mechanisms generate more than 10^7 different binding sites, and antigen specificity is maintained by processes that ensure that only one type of receptor is synthesized within any one cell. The production of antigen receptors occurs in the absence of antigen. Therefore, a diverse repertoire of antigen receptors is available before antigen is seen.

Although they share similar structural features, the surface antibodies on B cells and the T-cell receptors found on T cells are encoded by separate gene families; their expression

is cell-type specific. The surface antibodies on B cells can bind to soluble antigens, while the T-cell receptors recognize antigens only when displayed on the surface of other cells.

When B-cell surface antibodies bind antigen, the B lymphocyte is activated to secrete antibody and is stimulated to proliferate. T cells respond in a similar fashion. This burst of cell division increases the number of antigen-specific lymphocytes, and this clonal expansion is the first step in the development of an effective immune response. As long as the antigen persists, the activation of lymphocytes continues, thus increasing the strength of the immune response. After the antigen has been eliminated, some cells from the expanded pools of antigen-specific lymphocytes remain in circulation. These cells are primed to respond to any subsequent exposure to the same antigen, providing the cellular basis for immunological memory.

In the first step in mounting an immune response the antigen is engulfed by an antigen presenting cell (APC). The APC degrades the antigen and pieces of the antigen are presented on the cell surface by a glycoprotein known as the major histocompatibility complex class II proteins (MHC II). Helper T-cells bind to the APC by recognizing the antigen and the class II protein. The protein on the T-cell which is responsible for recognizing the antigen and the class II protein is the T-cell receptor (TCR).

Once the T-cell binds to the APC, in response to Interleukin I and II (IL), helper T-cell proliferate exponentially. In a similar mechanism, B cells respond to an antigen and proliferate in the immune response.

The TCR acts in conjunction with a protein that is also expressed on the surface of the T-cell called CD3. The complex is the TCR-CD3 complex. Depending on the type of lymphocyte, the lymphocyte can also express other cell surface proteins which include CD2, CD4, CD8, and CD45. The interactions between these cell surface proteins are important in the stimulation of T cell response.

Two major sub-populations of T cells have been identified. CD4 lymphocytes can present on its cell surface, the CD4 protein, CD3 and its respective T cell receptor. CD8 lymphocytes can present on its cell surface, the CD8 protein, CD3 and its respective T cell receptor.

CD4 lymphocytes generally include the T-helper and T-delayed type hypersensitivity subsets. The CD4 protein typically interacts with Class II major histocompatibility complex. CD4 may function to increase the avidity between the T cell and its MHC class II APC or stimulator cell and enhance T cell proliferation.

CD8 lymphocytes are generally cytotoxic T-cells, whose function is to identify and kill foreign cells or host cells displaying foreign antigens. The CD8 protein typically interacts with Class I major histocompatibility complex.

IV. Clinical Use Of Antibodies.

Clinical trials of the ATG treatment suggested a significant reduction of early rejection episodes, improved long term survival and, most importantly, reversal of ongoing rejection episodes. However, the results were often inconsistent due to the inability to standardize individual preparations of antisera. In addition, the precise nature of the target antigens recognized by the polyclonal reagents could not be defined, thus making scientific analysis difficult. The advent of monoclonal antibody (mAb) technology provided the bases for developing potentially therapeutic reagents that react with specific cell surface antigens which are involved in T cell activation.

One of the clinically successful uses of monoclonal antibodies is to suppress the immune system, thus enhancing the efficacy of organ or tissue transplantation. U.S. Patent 4,658,019, describes a novel hybridoma (designated OKT3) which is capable of producing a monoclonal antibody against an antigen found on essentially all normal human peripheral T cells. This antibody is said to be monospecific for a single determinant on these T cells, and does not react with other normal peripheral blood lymphoid cells. The OKT3 mAb described in this patent is currently employed to prevent renal transplant rejection (Goldstein, 1987).

In addition, other cell surface molecules have been identified that can activate T cell function, but are not necessarily part of the T cell surface receptor complex. Monoclonal antibodies against Thy-1, TAP, Ly-6, CD2, or CD28 molecules can activate T cells in the absence of foreign antigen *in vitro*. Moreover, certain bacterial proteins although differing in structure from mAbs, also have been shown to bind to subsets of T cells and activate them *in vitro*.

The possibility of selectively down-regulating the host's immune response to a given antigen represents one of the most formidable challenges of modern immunology in relation to the development of new therapies for IgE-mediated allergies, autoimmune diseases and the prevention of immune rejection of organ transplants. Similar considerations apply to an increasing number of promising therapeutic modalities for a broad spectrum of diseases, which would involve the use of foreign biologically active agents potentially capable of modulating the immune response, provided they were not also immunogenic. Among these agents, one may cite xenogeneic monoclonal or polyclonal antibodies (collectively referred to here as xIg) against different epitopes of the patients' $CD4^+$ cells (Diamantstein 1986), administered alone or in combination with immunosuppressive drugs for the treatment of rheumatoid arthritis and other autoimmune diseases, or for the suppression of graft-versus-host reactions and the immune rejection of organ transplants.

The therapeutic effectiveness of these immunological strategies is undermined by the patients' antibodies which prevent these bullets from reaching their target cells. In addition, the repeated administration of these agents may result in serious complications, viz. serum sickness, anaphylactic symptoms (*i.e.,* bronchospasm, dyspnea and hypotension) and/or the deposition in the liver of toxic immune complexes leading frequently to hepatotoxicity.

V. Preparation Of Monoclonal And Polyclonal Antibodies.

The methods for generating monoclonal antibodies (MAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen, and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of

antisera. Typically an animal used for production of anti-antisera is a rabbit, a mouse, a rat, a hamster or a guinea pig. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given polypeptide or polynucleotide may vary in its immunogenicity. It is often necessary therefore to couple the immunogen with a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers.

Means for conjugating a polypeptide or a polynucleotide to a carrier protein are well known in the art and include glutaraldehyde, m-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As is also well known in the art, immunogencity to a particular immunogen can be enhanced by the use of non-specific stimulators of the immune response known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant, incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen used of the production of polyclonal antibodies varies *inter alia,* upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal. The production of polyclonal antibodies is monitored by sampling blood of the immunized animal at various points following immunization. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored.

A monoclonal antibody of the present invention can be readily prepared through use of well-known techniques such as those exemplified in U.S. Patent 4,196,265, herein incorporated by reference. Typically, a technique involves first immunizing a suitable animal with a selected antigen (*e.g.*, a polypeptide or polynucleotide of the present invention) in a

manner sufficient to provide an immune response. Rodents such as mice and rats are preferred animals. Spleen cells from the immunized animal are then fused with cells of an immortal myeloma cell. Where the immunized animal is a mouse, a preferred myeloma cell is a murine NS-1 myeloma cell.

The fused spleen/myeloma cells are cultured in a selective medium to select fused spleen/myeloma cells from the parental cells. Fused cells are separated from the mixture of non-fused parental cells, for example, by the addition of agents that block the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides. Where azaserine is used, the media is supplemented with hypoxanthine.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants for reactivity with an antigen-polypeptides. The selected clones can then be propagated indefinitely to provide the monoclonal antibody.

By way of specific example, to produce a monoclonal antibody, mice are injected intraperitoneally with between about 1-200 μg of an antigen comprising a polypeptide of the present invention. B lymphocyte cells are stimulated to grow by injecting the antigen in association with an adjuvant such as complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis)*. At some time (*e.g.*, at least two weeks) after the first injection, mice are boosted by injection with a second dose of the antigen mixed with incomplete Freund's adjuvant.

A few weeks after the second injection, mice are tail bled and the sera titered by immunoprecipitation against radiolabeled antigen. Preferably, the process of boosting and

titering is repeated until a suitable titer is achieved. The spleen of the mouse with the highest titer is removed and the spleen lymphocytes are obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5 X 10^7 to 2 X 10^8 lymphocytes.

Mutant lymphocyte cells known as myeloma cells are obtained from laboratory animals in which such cells have been induced to grow by a variety of well-known methods. Myeloma cells lack the salvage pathway of nucleotide biosynthesis. Because myeloma cells are tumor cells, they can be propagated indefinitely in tissue culture, and are thus denominated immortal. Numerous cultured cell lines of myeloma cells from mice and rats, such as murine NS-1 myeloma cells, have been established.

Myeloma cells are combined under conditions appropriate to foster fusion with the normal antibody-producing cells from the spleen of the mouse or rat injected with the antigen/polypeptide of the present invention. Fusion conditions include, for example, the presence of polyethylene glycol. The resulting fused cells are *hybridoma* cells. Like myeloma cells, hybridoma cells grow indefinitely in culture.

Hybridoma cells are separated from unfused myeloma cells by culturing in a selection medium such as HAT media (hypoxanthine, aminopterin, thymidine). Unfused myeloma cells lack the enzymes necessary to synthesize nucleotides from the salvage pathway because they are killed in the presence of aminopterin, methotrexate, or azaserine. Unfused lymphocytes also do not continue to grow in tissue culture. Thus, only cells that have successfully fused (hybridoma cells) can grow in the selection media.

Each of the surviving hybridoma cells produces a single antibody. These cells are then screened for the production of the specific antibody immunoreactive with an antigen/polypeptide of the present invention. Single cell hybridomas are isolated by limiting dilutions of the hybridomas. The hybridomas are serially diluted many times and, after the dilutions are allowed to grow, the supernatant is tested for the presence of the monoclonal

antibody. The clones producing that antibody are then cultured in large amounts to produce an antibody of the present invention in convenient quantity.

VI. Engineering Antibodies for Use in the Present Invention

The present invention provides antibodies to be used as treatments for graft rejection and other autoimmune diseases. It is known that the murine antibody OKT3 is a powerful immunosuppressive agent. However, the provision of murine OKT3 to patients experiencing, for example graft rejection, is hampered by a first-dose reaction that renders further doses of OKT3 ineffective. The activating properties of OKT3 have been attributed to T cell activation by the mAb which results in TcR cross-linking. Thus, before the OKT3 can mediate immunosuppression, it triggers activation of mAb-bound T cells and FctR-bearing cells, resulting in a massive systemic release of cytokines responsible for the acute toxicity of the mAb (Abramowicz, 1989; Chatenoud, 1989). The present invention demonstrates that the absence of FcR binding capacity of anti-CD3 allows the mAbs to retain immunosuppressive properties, without being hampered by the mitogenic effects.

In order to achieve these objectives, it is possible to engineer antibodies. In the first instance, it is possible to produce humanized antibodies that will have a reduced immune response as compared to the murine antibody. Secondly, in order disrupt or abrogate the first-dose reactions attributed to the T cell activation by the murine mAb, it will be possible to make mutants of both the humanized and original murine antibody that lack the FcR binding domain and therefore avoid the toxicity and immunization induced by OKT3.

a. General Methods of Engineering Antibodies

Methods of engineering antibodies to have an altered structure or function are well known to those of skill in the art. For example, in U.S. Patent 5,648,260 (specifically incorporated herein by reference) Winter, *et. al.* describe methods and compositions comprising DNA encoding an antibody with an altered function, e.g. altered affinity for an Fc receptor (FcR). The composition is produced by replacing the nucleic acid encoding at least one amino acid residue in a given portion of the antibody with nucleic acid encoding a different residue.

It possible to clone DNA encoding a specific antibody's heavy and light chains, and to express the cloned antibody chains following transfection into eukaryotic cells (Neuberger *et al.*, 1983; Gilles *et al.*, 1983). Like hybridomas, these transfected cells can be selected, screened and cloned as stable transfectomas that secrete a monoclonal antibody (Sharon *et al.*, 1984; Morrison, 1985). In addition, the ability to clone the DNA of individual portions of a gene segment (Larrick *et al.*, 1989; Orlandi *et al.*, 1989; Heinrichs *et al.*, 1995) and to manipulate these domain segments by specific mutation and random combination has facilitated the engineering of "artificial" antibody combining sites to a variety of epitopes that can be expressed in transfectomas or by phage display (McMafferty *et al.*, 1990; Winter and Milstein, 1991; Huston *et al.*, 1988; Yamanaka *et al.*, 1996)). These techniques have proved successful in producing monoclonal antibody-type reagents with new specificities and with modified effector functions.

The amino acid and nucleotide sequences for murine OKT3 are given in SEQ ID NOS: 2-5 and 1. Given that the native sequence is know it will be possible to create mutants using teachings well known to those of skill in the art and described herein. A particular aspect of the present invention contemplates generating mutants of the OKT3 antibody that diminish the FcR binding capacity of the antibody whilst retaining its immunosuppressive capabilities. Such mutants will have use in the therapeutic applications of the present invention.

i. Amino acid Variants

Amino acid sequence variants of the antibody polypeptide can be created such that they are substitutional, insertional or deletion variants. Deletion variants lack one or more residues of the native protein which are not essential for function or immunogenic activity. Insertional mutants typically involve the addition of material at a non-terminal point in the polypeptide. This may include the insertion of an immunoreactive epitope or simply a single residue. Terminal additions, called fusion proteins, are discussed below.

Substitutional variants typically contain the exchange of one amino acid for another at one or more sites within the protein, and may be designed to modulate one or more properties of the polypeptide, such as stability against proteolytic cleavage, without the loss of other functions or properties. Substitutions of this kind preferably are conservative, that is, one amino acid is replaced with one of similar shape and charge. Conservative substitutions are well known in the art and include, for example, the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine.

Once general areas of the gene are identified as encoding particular protein domains as described herein below, point mutagenesis may be employed to identify with particularity which amino acid residues are important in particular activities associated with a particular function. Thus, one of skill in the art will be able to generate single base changes in the DNA strand to result in an altered codon and a missense mutation.

The following is a discussion based upon changing of the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid substitutions can be made in a protein sequence, and its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the DNA sequences of genes without appreciable loss of their biological utility or activity, as discussed below. Table 1 shows the codons that encode particular amino acids.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte & Doolittle, 1982). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte & Doolittle, 1982), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.*, still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

TABLE 1

Amino Acids			Codons
Alanine	Ala	A	GCA GCC GCG GCU
Cysteine	Cys	C	UGC UGU
Aspartic acid	Asp	D	GAC GAU
Glutamic acid	Glu	E	GAA GAG
Phenylalanine	Phe	F	UUC UUU
Glycine	Gly	G	GGA GGC GGG GGU
Histidine	His	H	CAC CAU
Isoleucine	Ile	I	AUA AUC AUU
Lysine	Lys	K	AAA AAG
Leucine	Leu	L	UUA UUG CUA CUC CUG CUU
Methionine	Met	M	AUG
Asparagine	Asn	N	AAC AAU

TABLE 1 Continued

Amino Acids			Codons					
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine *-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent and immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those that are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 nucleotides on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art. As will be appreciated, the technique typically employs a bacteriophage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage vectors are commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis, which eliminates the step of transferring the gene of interest from a phage to a plasmid.

In general, site-directed mutagenesis is performed by first obtaining a single-stranded vector, or melting of two strands of a double stranded vector which includes within its sequence a DNA sequence encoding the desired protein. An oligonucleotide primer bearing the desired mutated sequence is synthetically prepared. This primer is then annealed with the single-stranded DNA preparation, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected gene using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting, as there are other ways in which sequence variants of genes may be obtained. For example, recombinant vectors encoding the desired gene may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

ii. Expression Vectors for Encoding Mutants

Within certain embodiments expression vectors are employed to express various genes to encode a specific antibody, which can then be purified and, be used to generate antisera or monoclonal antibody with which further studies may be conducted. Expression requires that appropriate signals be provided in the vectors, and which include various regulatory elements, such as enhancers/promoters from both viral and mammalian sources that drive expression of the genes of interest in host cells.

Elements designed to optimize messenger RNA stability and translatability in host cells also are defined. The conditions for the use of a number of dominant drug selection markers for establishing permanent, stable cell clones expressing the polypeptide products are also provided, as is an element that links expression of the drug selection markers to expression of the polypeptide.

Regulatory Elements. Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. In certain embodiments, expression includes both transcription of a gene and translation of mRNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid encoding a gene of interest.

In preferred embodiments, the nucleic acid encoding a gene product is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by

the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies amongst others, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter employed to control the expression of a nucleic acid sequence of interest is not believed to be important, so long as it is capable of direction the expression of the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable

to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose.

By employing a promoter with well-known properties, the level and pattern of expression of the protein of interest following transfection or transformation can be optimized. Further, selection of a promoter that is regulated in response to specific physiologic signals can permit inducible expression of the gene product. The present application lists several elements/promoters which may be employed, in the context of the present invention, to regulate the expression of the gene of interest. This list is not intended to be exhaustive of all the possible elements involved in the promotion of gene expression but, merely, to be exemplary thereof.

Enhancers are genetic elements that increase transcription from a promoter located at a distant position on the same molecule of DNA. Enhancers are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular

orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Below is a list of viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the nucleic acid encoding a gene of interest in an expression construct. Additionally, any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of the gene. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct. Enhancer/promoter elements contemplated for use with the present invention include but are not limited to Immunoglobulin Heavy Chain, Immunoglobulin Light, Chain T-Cell Receptor, HLA DQ α and DQ β, β-Interferon, Interleukin-2, Interleukin-2 Receptor, MHC Class II 5, MHC Class II HLA-DRα, β-Actin, Muscle Creatine Kinase, Prealbumin (Transthyretin), Elastase *I,* Metallothionein, Collagenase, Albumin Gene, α-Fetoprotein, τ-Globin, β-Globin, e-fos, c-HA-ras, Insulin, Neural Cell Adhesion Molecule (NCAM), α1-Antitrypsin, H2B (TH2B) Histone, Mouse or Type I Collagen, Glucose-Regulated Proteins (GRP94 and GRP78), Rat Growth Hormone, Human Serum Amyloid A (SAA), Troponin I (TN I), Platelet-Derived Growth Factor, Duchenne Muscular Dystrophy, SV40, Polyoma, Retroviruses, Papilloma Virus, Hepatitis B Virus, Human Immunodeficiency Virus, Cytomegalovirus, Gibbon Ape Leukemia Virus. Inducible promoter elements and their associated inducers are also contemplated.

In certain embodiments of the invention, the expression construct comprises a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells via receptor-mediated endocytosis, to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and Sugden, 1986; Temin, 1986). The first viruses used as gene vectors were DNA viruses including the papovaviruses (simian virus 40, bovine papilloma virus, and polyoma) (Ridgeway, 1988; Baichwal and Sugden, 1986) and adenoviruses (Ridgeway, 1988; Baichwal and Sugden, 1986). These have a relatively low capacity for foreign DNA sequences and

have a restricted host spectrum. Furthermore, their oncogenic potential and cytopathic effects in permissive cells raise safety concerns. They can accommodate only up to 8 kB of foreign genetic material but can be readily introduced in a variety of cell lines and laboratory animals (Nicolas and Rubenstein, 1988; Temin, 1986).

Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

Selectable Markers. In certain embodiments of the invention, the cells contain nucleic acid constructs of the present invention, a cell may be identified *in vitro* or *in vivo* by including a marker in the expression construct. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression construct. Usually the inclusion of a drug selection marker aids in cloning and in the selection of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. Alternatively, enzymes such as herpes simplex virus thymidine kinase (*tk*) or chloramphenicol acetyltransferase (CAT) may be employed. Immunologic markers also can be employed. The selectable marker employed is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable markers are well known to one of skill in the art.

Multigene constructs and IRES. In certain embodiments of the invention, the use of internal ribosome binding sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5′ methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picanovirus family (polio and

encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

Any heterologous open reading frame can be linked to IRES elements. This includes genes for secreted proteins, multi-subunit proteins, encoded by independent genes, intracellular or membrane-bound proteins and selectable markers. In this way, expression of several proteins can be simultaneously engineered into a cell with a single construct and a single selectable marker.

iii. Delivery of Genetic Constructs

In order to effect expression of gene constructs, the expression construct must be delivered into a cell. This delivery may be accomplished *in vitro*, as in laboratory procedures for transforming cells lines, or *in vivo* or *ex vivo*, as in the treatment of certain disease states. One mechanism for delivery is via viral infection where the expression construct is encapsidated in an infectious viral particle.

Viral delivery may be achieved using an adenovirus expression vector (Grunhaus and Horwitz, 1992; Renan 1990; Graham and Prevec, 1991), retroviruses (Coffin, 1990; Roux *et al.,* 1989), as well as other viral vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.,* 1988) adeno-associated virus (AAV) (Ridgeway, 1988; Baichwal and Sugden, 1986; Hermonat and Muzycska, 1984) and herpes viruses may be employed. These viral vectors offer several attractive features for various mammalian cells (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.,* 1988; Horwich *et al.,* 1990).

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention. These include calcium

phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979) and lipofectamine-DNA complexes, cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

Once the expression construct has been delivered into the cell the nucleic acid encoding the gene of interest may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding the gene may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation via homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

In one embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is particularly applicable for transfer *in vitro* but it may be applied to *in vivo* use as well. Dubensky *et al.*, (1984) successfully injected polyomavirus DNA in the form of calcium phosphate precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of calcium phosphate-precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding a gene of interest may also be transferred in a similar manner *in vivo* and express the gene product.

Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

In a further embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated are lipofectamine-DNA complexes.

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression constructs have been successfully employed in transfer and expression of nucleic acid *in vitro* and *in vivo*, then they are applicable for the present invention. Where a bacterial promoter is employed in the DNA construct, it also will be desirable to include within the liposome an appropriate bacterial polymerase.

The gene for the protein of interest may be transferred as described above into appropriate host cells followed by culture of cells under the appropriate conditions. Primary

animal cell cultures for generating the antibody polypeptide may be prepared in various ways. In order for the cells to be kept viable while *in vitro* and in contact with the expression construct, it is necessary to ensure that the cells maintain contact with the correct ratio of oxygen and carbon dioxide and nutrients but are protected from microbial contamination. Cell culture techniques are well documented and are disclosed herein by reference (Freshner, 1992).

b. Specific "Humanized" Anti-CD3 Monoclonal Antibodies.

In order to improve the effectiveness and expand the human uses of anti-CD3 antibodies such as for example, OKT3, humanized versions of the antibody have been generated. The skilled artisan is referred to U.S. Patent Application Serial Number 08/557,050 and allowed U.S. Patent Application Serial No. 08/070,116 (each document is specifically incorporated by reference in its entirety) which describe specific humanized anti-CD3 antibodies. A particular anti-CD3 antibody useful in the present invention is OKT3, although other useful antibodies can be constructed with the methods disclosed herein. More specific examples of methods of making humanized (and/or non-mitogenic) OKT3 are given in the Examples herein below. Although the discussion herein below refers to OKT3, the techniques are equally applicable to all other antibodies, and will be useful in generating non-OKT3 based anti-CD3 antibodies and other antibodies for therapeutic applications.

It has been shown (Woodle, 1992) that simple transfer of the loop regions and the complementarity determining regions (CDR's) (Kabat, 1987), which are believed to contain the antigen contacting amino acids, into a human framework was not sufficient in the case of OKT3 to provide the structure required for efficient antigen binding. Examination of the remaining framework residues identified several which could potentially contribute to a reconstitution of binding in a human framework. When amino acids at these positions in the human framework were replaced with those from OKT3 to give gOKT3-5, antigen binding was shown to be fully restored. Subsequently, it has been noted (Woodle *et al*, 1991) that a number of these amino acids derived from the OKT3 sequence are not required to achieve a humanized antibody with the same affinity as murine OKT3.

To reduce the immune responses observed in patients treated with murine OKT3, a "humanized" OKT3 (gOKT3-5), comprised of the complementary determining regions (CDR) of the murine anti-CD3 mAb and of the variable framework and constant regions of a human IgG4, was developed. General methods for producing humanized antibodies are discussed in US Patents 5,646,253; 5,225,539; 5,624,821; 5,693,762 (each specifically incorporated herein by reference).

c. Point Mutations in "Humanized" Monoclonal Antibodies.

As a therapeutic drug, a major problem associated with OKT3 is the first-dose reactions attributed to the T cell activation by the mAb. These properties are not removed by forming a humanized OKT3 monoclonal antibody. Since gOKT3-5 produces, *in vitro*, similar activation to OKT3, it is quite likely that the same side-effects might also occur with this drug *in vivo*. $F(ab')_2$ fragments of OKT3 have led to potent immunosuppression and TCR modulation, *in vitro*. Non-activating $F(ab')_2$ fragments of anti-CD3 mAbs to mice was as efficacious as whole anti-CD3 in delaying skin graft rejection, while the $F(ab')_2$ fragments exhibited significantly reduced T cell activation and fewer side-effects in mice. However, the production of $F(ab')_2$ fragments in large quantities remains difficult. Furthermore, the half-life of this drug in the blood stream is relatively short, as compared with whole mAb. Thus, frequent injections of the $F(ab')_2$ fragments of anti-CD3 were necessary to achieve maximal immunosuppression, making the use of this mAb fragment inappropriate for clinical transplantation. Finally, recent studies have shown that even a small contaminant of whole mAb in the $F(ab')_2$ preparation ($<1/10^4$ molecules) has a synergistic effect on T cell activation.

The Fc portion of the murine IgG2a Abs, including OKT3, binds preferentially to the high affinity 72 kD FcR I (CD64) present on human macrophages and IFN-γ-stimulated polymorphonuclear leukocytes (Anderson, 1986; Lynch, 1990; Shen, 1987), but also to the low affinity 40 kD FcR II (CD32) that is found on human macrophages, ß cells and polymorphonuclear neutrophils (Anderson, 1986; Petroni, 1988; Bentin, 1991). The CH2 region in the Fc portion of IgGs has been found to be the domain that selectively binds FcR I and II (Ollo, 1983; Woof, 1984; Burton, 1985; Partridge, 1986; Duncan, 1988). In fact, the

exact binding segment has been localized to an area corresponding to amino acids 234 to 238 (Duncan, 1988) and the respective affinity of several isotypes has been determined (Gergely, 1990).

Duncan *et al.* have shown that the mutation of a single amino acid in the FcR binding segment of a murine IgG2b, converting the sequence to that found in a murine IgG2a, resulted in a 100-fold enhancement of the binding to FcR (1988). Based on those data, a mutation was introduced into the Fc region of an anti-CD3 human IgG4 antibody resulting in a sequence similar to the low affinity sequence of the murine IgG2b. This mAb contains a glutamic acid rather than a leucine at position 235 of the human IgG4 heavy chain (Glu-235 mAb). The mutational analysis was performed on a "humanized" anti-CD3 mAb, the gOKT3-5 mAb by splicing the murine complementarily determining regions into the human IgG4 framework gene sequence.

The gOKT3-5 mAb was previously shown to retain binding affinity for the CD3 complex similar to murine OKT3 and all the *in vitro* activation and immunosuppressive properties of OKT3. In addition, the gOKT3-5 mAb had an FcR binding sequence differing by only two amino acids from the same region on the murine IgG2b or by one amino acid in the murine IgG2a/human IgG1. Since a mutation in the FcR binding region of the mAb could modify the conformation of the molecule and thus be responsible for a decrease in FcR binding regardless of the amino acid sequence obtained, a control mutation of amino acid 234 from a phenylalanine into a leucine was performed in order to mimic the FcR binding area found in the high affinity murine IgG2a and human IgG1. This mAb was designated Leu-234.

Therefore, the site-specific mutations described above were introduced into the Fc portion of the gOKT3-5 mAb to affect the binding of the Ab to FcR. The appropriate mutant of the anti-CD3 mAb was designed to exhibit the low-activating properties of $F(ab')_2$ fragments, the purity of a monoclonal antibody and an increased serum half-life as compared with $F(ab')_2$ fragments or possibly even with murine OKT3, since chimeric mouse/human antibodies have been shown to circulate longer their murine counterpart. The resulting mAb

thus avoids the acute toxicity and the immunization induced by OKT3, *in vivo*, although, theoretically, the substitution of glutamic acid at position 235 in order to mimic murine IgG2b could also create an immunogenic epitope in the constant region of the humanized antibody.

In fact, a single amino acid substitution of a glutamic acid for a leucine at position 235 in the Fc portion of the gOKT3-5 mAb resulted in a mAb which bound U937 cells 100-fold less than the murine OKT3. This mutation, which generated an FcR binding sequence similar to the one found in murine IgG2b, resulted in a mAb with a 10-fold lower affinity for FcR than the murine IgG2b. The reason for this difference is unclear but may imply that the interaction of the five amino acid-FcR binding region with the adjacent amino acids, which in the case of the Glu mAb are part of a human IgG4, is relevant to FcR binding.

All the Abs tested showed some modulation of the TCR after a culture of 12 hours. However, the Glu-235 mAb had to be added in higher concentrations or for a longer period of time to achieve maximal modulation. This suggests that low FcR binding might delay the induction of TCR internalization. All the Abs also inhibited CTL activity, indicating similar suppressive properties by this assay. Thus, altering the binding of the gOKT3-5 mAb by site-directed mutagenesis did not significantly affect the immunosuppressive ability of the mAb, *in vitro*.

The reduced binding of the Glu-235 mAb correlated with a marked decrease in the T cell activation induced by this Ab, as assessed by the absence of T cell proliferation, the decreased expression of cell surface markers of activation, the diminished release of TNF-α and GM-CSF and the lack of secretion of IFN-γ. The magnitude of T cell mitogenesis is known to correlate with the affinity of anti-CD3 mAbs for FcR I, whose relative binding is IgG1=IgG3>IgG4 for human subclasses of Abs and IgG2a=IgG3>IgG1>IgG2b for murine isotypes. The anti-CD3 mAbs employed in this study displayed an FcR binding as expected, with the human IgG4 gOKT3-5 mAb binding less avidly to U937 cells than murine IgG2a OKT3 or Leu-234 mAb, but with much higher affinity than the Glu-235 mAb.

The activation induced by the different anti-CD3 mAbs tested did not entirely correlate with their affinity for FcRs. In spite of the increased affinity of OKT3 for FcRs as compared with the gOKT3-5 mAb, no significant difference in the T cell activation was observed between the two mAbs. One explanation could be that activation is maximal whenever a certain threshold of cross-linking between T lymphocytes and FcR is attained. Another possibility is that the binding of the mAb to the CD3 antigen potentiates its avidity for FcR-bearing cells.

The extent of the functional changes generated in the FcR binding region of the gOKT3-5 mAb that form the Glu-235 mAb has further implications. The ability of certain isotypes of anti-CD3 mAbs to activate T cells and mediate ADCC has been shown to vary in the population. Murine IgG2a and IgG3 anti-CD3 mAbs are mitogenic for virtually all individuals. In contrast, murine IgG1 and IgG2b mAbs induce proliferation in only 70% and 5% to 10%, respectively. The Glu mAb, which appears to function as a non-activator IgG2b in a small fraction of the population. However, even in these individuals, IgG2b mAbs seen to trigger a different pathway of activation. For instance, in contrast to other anti-CD3 isotypes, IgG2b mAbs do not induce the production of IL-2 or IFN-γ. Thus, the proliferation observed in the small subset of the patient population may be an IL-2 independent T cell mitogenesis, which has previously been reported in other settings. More importantly, the reduced FcR binding of the Glu-235 mAb to FcR, as compared with murine IgG2b Abs, may be sufficient to abrogate the activation of even this subset of individuals.

In one embodiment, the present invention contemplates a class of homo-bifunctional antibodies, a humanized version of OKT3 which also interacts with CD4. This humanized antibody has an Fv region containing the CD3 ε antigen specificity of OKT3 and an Fc region from either human IgG1 or IgG4 antibody. The humanized anti CD3 antibody binds CD4 directly, either immobilized on plastic or on $CD4^+$, $CD3^-$, FcR cells.

Initial mapping experiments suggest that the binding occurs near the OKT4A epitope on CD4. The weak interaction of some antibodies (but not human IgG4) with this region of CD4, independent of antigen/antibody binding site, has been reported (Lanert, 1991).

However, unlike these reports, the antibody of the present invention binds with either a γ1 or a γ4 heavy chain. The CD4 binding site on humanized OKT3 has been mapped to the Fab fragment and probably resides in the framework sequences of the variable region.

By use of a monoclonal antibody of the present invention, specific polypeptides and polynucleotides of the invention can be recognized as antigens, and thus identified. Once identified, those polypeptides and polynucleotides can be isolated and purified by techniques such as antibody-affinity chromatography. In antibody-affinity chromatography, a monoclonal antibody is bound to a solid substrate and exposed to a solution containing the desired antigen. The antigen is removed from the solution through an immunospecific reaction with the bound antibody. The polypeptide or polynucleotide is then easily removed from the substrate and purified.

VII. Protein purification.

It will be desirable to purify antibody once it has been produced by the techniques described herein above. Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level, the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Having separated the polypeptide from other proteins, the polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion chromatography; polyacrylamide gel electrophoresis; isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even HPLC.

Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an encoded protein or peptide. The term "purified protein or peptide" as used herein, is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state. A purified protein or peptide therefore also refers to a protein or peptide, free from the environment in which it may naturally occur.

Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a "-fold purification number." The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general

purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater "-fold" purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

VIII. Pharmaceutical Compositions.

In a preferred embodiment, the present invention provides pharmaceutical compositions comprising antibodies immunoreactive with CD3 and CD4 cell surface antigens.

A composition of the present invention is typically administered parenterally in dosage unit formulations containing standard, well-known nontoxic physiologically acceptable carriers, adjuvants, and vehicles as desired. The term parenteral as used herein includes intravenous, intramuscular, intraarterial injection, or infusion techniques.

Graft rejection and other diseased states requiring immunosuppression, such as for example, any of a variety of autoimmune diseases (e.g. systemic lupus erythematosus (SLE), progressive systemic scleroderma, mixed connective tissue disease and antiphospholipid syndrome or any other immune disease requiring anti-CD3 mediated immune suppression) may be treated with a combination therapeutic approach. In such an instance the FcR nonbinding anti-CD3 antibody may be combined with another immunosuppressant such as cyclosporin A or FK506, or any agent derived therefrom.

Various combinations may be employed as described herein below where the FcR nonbinding anti-CD3 antibody is "A" and the immunosuppressive is "B":

A/B/A B/A/B B/B/A A/A/B A/B/B B/A/A A/B/B/B B/A/B/B

B/B/B/A B/B/A/B A/A/B/B A/B/A/B A/B/B/A B/B/A/A

B/A/B/A B/A/A/B A/A/A/B B/A/A/A A/B/A/A A/A/B/A

The terms "contacted" and "exposed," when applied to a cell, are used herein to describe the process by which an antibody of the present invention and an immunosuppressive agent such as CsA are delivered to a target cell or are placed in direct juxtaposition with the target cell. To achieve immunosuppression, both agents are delivered to the cell in a combined amount effective to achieve immunosuppression without a concomitant anti-CD3 mediated mitogenicity. As used in the present context the cell may be part of a skin graft or a renal transplant and the like. The therapeutic composition(s) may be delivered regionally to the area of the graft or may be administered systematically.

Injectable preparations, for example sterile injectable aqueous or oleaginous suspensions, are formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation can also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol.

Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

Preferred carriers include neutral saline solutions buffered with phosphate, lactate, Tris, and the like. Of course, one purifies the vector sufficiently to render it essentially free of undesirable contaminant, such as defective interfering adenovirus particles or endotoxins and other pyrogens such that it does not cause any untoward reactions in the individual receiving the vector construct. A preferred means of purifying the vector involves the use of buoyant density gradients, such as cesium chloride gradient centrifugation.

A carrier can also be a liposome. Means for using liposomes as delivery vehicles are well known in the art (*See, e.g.*, Gabizon *et al.*, 1990; Ferruti *et al.*, 1986). Liposomal compositions have previously been described above for the production of recombinant antibodies, the teachings described above for the use of liposomes to transfer DNA into a cell are also applicable for using liposomes to carry therapeutic compositions to a cell.

In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau *et al.*, (1987) employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. Thus, it is feasible that a nucleic acid encoding a particular gene also may be specifically delivered into a cell type such as lung, epithelial or tumor cells, by any number of receptor-ligand systems with or without liposomes. For example, epidermal growth factor (EGF) may be used as the receptor for mediated delivery of a nucleic acid encoding a gene in many tumor cells that exhibit upregulation of EGF receptor. Mannose can be used to target the mannose receptor on liver cells. Also, antibodies to CD5 (CLL), CD22 (lymphoma), CD25 (T-cell leukemia) and MAA (melanoma) can similarly be used as targeting moieties.

A transfected cell can also serve as a carrier. By way of example, a liver cell can be removed from an organism, transfected with a polynucleotide of the present invention using methods set forth above and then the transfected cell returned to the organism (*e.g.* injected intravascularly).

IX. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed

and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Materials And Methods

Animals. 6-8 wk old BALB/c, DBA/2J, and B10.A mice may be purchased for example from Frederick Cancer Research Institute Laboratories (Frederick, MD). DO 11.10 mice, transgenic for an OVA peptide (323-339) specific, I-A^d restricted αβ-TCR, were a obtained from Drs. Dennis Loh and Ken Murphy (Washington U. in St. Louis, MO) (Murphy *et al.*, 1989) and the IL-4 knockout (KO) mice were obtained from Dr. S. Reiner (University of Chicago, Chicago, IL). All mice were bred and maintained in a specific pathogen free facility at the University of Chicago.

T-Cell Clones and Cell lines. The pigeon cytochrome *c*-specific Th1 clone, AB.7, may be obtained from Dr. M. Jenkins (University of Minnesota, Minneapolis, MN). The OVA-specific Th1 clone pGL10 and Th2 clone pL104 may be obtained from Dr. F. Fitch. pGL10, pL104, and AB.7 T cell clones were maintained as previously described except that the APC feeders for pL104 were irradiated at 3000 rad (Stack *et al.*, 1994; Quill and Schwartz, 1987). In some experiments, Th0 clones (24.5 and 4.5) derived from the DO 11.10 TCR transgenic were also obtained from Dr. Fitch. These clones were maintained by restimulation every 7 to 14 days with 0.2 mg/ml OVA peptide, 12.5 U/ml rIL-2, and irradiated (3000 rad) H-2^d splenic APC.

Mixed T cell lines were generated as follows: In a 24-well dish, 1 to 1.5×10^5 DO 11.10 lymph node cells per well were activated with 0.3 to 1 μg/ml of OVA peptide in the presence of 6×10^6 irradiated (2000 rad) H-2^d splenic APC and 12.5 U/ml of IL-2 for 8 to 12 days before challenge with anti-CD3 IgG3. For the IL-4KO and IFN-γ KO lines, lymph node cells were CD8-depleted with the 3.155 mAb and complement, then 5×10^5 cells per well were stimulated with 0.03 to 0.1 μg/ml anti-CD3 (145-2C11) and 4.5 to 5×10^6 anti-Thy-1 T-depleted irradiated II-2^d splenocytes for 7 to 12 days. In subsequent rounds of cytokine KO T cell stimulation, 1×10^5 cells were plated per well. Similar results were obtained from first

round cultures with non-CD8-depleted lymph node cells. All T cell lines were restimulated every 7 to 14 days.

Antibodies and Reagents.

The following mAbs were used in this study: 145-2C11 (anti-CD3), AT83A (anti-Thy-1) [prepared in the inventors' laboratory]; anti-CD3-IgG3 (Alegre *et al.*, 1995); the anti-Ig antisera: goat anti-mouse IgG3 (Sigma, St. Louis, MO), rabbit anti-mouse IgG3 (Zymed, San Francisco, CA), rabbit anti-hamster (Cappel, Durham, NC); 145-2C11-FITC (Boehringer Mannheim, Indianapolis, IN); PV-1 (anti-CD28) [may be obtained from Dr. Carl June, Naval Med. Res. Inst., MD]; H146 (anti–ζ mAb containing supernatant) [may be obtained from Dr. Frank Fitch, University of Chicago, Chicago, IL]; 4G10 (anti-phosphotyrosine) and anti-PLCγ-1 (mixed monoclonal Abs) [UBI, Lake Placid, NY]; 12-222 (anti-ZAP70 antiserum) [may be obtained from Dr. Arthur Weiss, UCSF, San Francisco, CA]. 3.155 (anti-CD8) mAb; 11B11 mAb (anti-IL-4) (may be obtained from Dr. E. Vitteta, University of Texas, Dallas, TX); S4B6 (anti-IL-2), 7D4 (anti-IL-2R), PC615.3 (anti-IL-2R), SFR8-B6 (anti-human HLA Bw6 rat control Ig) (anti-IL-2/IL-2R and rat control Ig are protein G-purified mAbs may be obtained from Dr. R. L. Hendricks, University of Illinois, Chicago, IL); H146 (anti-ζ) mAb supernatant (may be obtained from Dr. F. Fitch, University of Chicago, Chicago, IL); 4G10 (anti-phosphotyrosine) (UBI, Lake Placid, NY); 387 (anti-ζ) antiserum (may be obtained from Dr. L. Samelson, National Institutes of Health, Bethesda, MD); 1598-8 (anti-ZAP-70) antiserum (may be obtained from Dr. A. Weiss, University of California, San Francisco, CA); anti-active MAP kinase (Promega, Madison, WI), anti-ERK1 and ERK2 (Zymed, San Francisco, CA); anti-NF-ATc1 (Affinity BioReagents, Golden, CO); and goat anti-mouse FITC (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Goat serum was purchased from Vector Laboratories (Burlingame, CA). The OVA and pigeon cytochrome *c* Ag were purchased from Sigma and the rIL-2 was obtained from Cetus (San Francisco, CA). For certain studies, OVA peptide was obtained from Dr. Fitch. Low toxicity rabbit complement was purchased from Pel-Freez (Brown Deer, WI). Cyclosporine A was purchased from Sandoz (Basel, Switzerland). Indo-1 was purchased from Molecular Probes (Eugene, OR).

Anti-CD3-Fos x anti-CD4-Jun bispecific F(ab'-zipper)$_2$ production. The anti-CD3 antibody was derived from hamster anti-mouse CD3 hybridoma 145-2C11 (Leo *et al.*, 1987), and the anti-CD4 antibody from rat anti-mouse CD4 hybridoma GK1.5 (Dialynas *et al.*, 1983). The VH and VL sequences for 145-2C11 (Leo *et al.*, 1987), GenBank accession nos. U17870 and U17871) and GK1.5 (Dialynas *et al.*, 1983), had been determined. Homodimers of anti-CD3-Fos and anti-CD4-Jun (Fab'-zipper)$_2$ were expressed by the genetic method described by Kostelny *et al.* (1992). Anti-CD3-Fos and anti-CD4-Jun were individually purified from transfected Sp2/0 spent media by protein G Sepharose affinity chromatography (Carter *et al.*, 1992). The two homodimers were then reduced and reoxidized to form bispecific F(ab'-zipper)$_2$ as described (Kostelny *et al.*, 1992). Bispecific F(ab'-zipper)$_2$ was further purified by BAKERBOND Abx column chromatography or hydrophobic interaction chromatography on a Bio-Gel® Phenyl-5 PW column.

Proliferation Assays. pGL10 and AE.7 T cell clones were maintained by restimulation every 7-14 days with irradiated (2000 rads) DBA/2J spleen cells, 200μg/ml OVA and 12.5U/ml human recombinant IL-2. AE.7T cell clones were maintained by restimulation every 7-14 days with irradiated (3000 rads) B10.A spleen cells and 2μm PCC. Two days after plating spleen and antigen, each well of AE.7T cells was expanded into 4 wells and 10U/ml recombinant human IL-2 was added. T cell clones were purified by Ficoll Hypaque density centrifugation before use in all studies. For the cross-linking assays, whole BALB/c spleens were lysed in hypotonic ACK buffer to remove erythrocytes and washed in 5% fetal calf serum (FCS) supplemented with DMEM.. Proliferation and unresponsiveness assays were in 5% or 10% FCS supplemented DMEM. In a 96-well flat bottom plate, 2×10^5 splenocytes or 1×10^5 PGL10 T cells were incubated on ice for 10 minutes with anti-CD3 (final concentration of 1 μg/ml), and then for another 10 minutes on ice with the appropriate cross linker (rabbit anti-IgG3 at 1:30 or goat anti-IgG3 at 1:100 or goat anti-hamster at 1:300), before being placed at 37°C. The amount of cross-linker used for these assays and the biochemical studies was determined by titration to yield maximum T cell proliferation. For anti-CD3fos or anti-CD3 × anti-CD4 proliferation assays, antibodies were serially diluted in 96 well flat bottom plates, starting at 10 μg/ml. Assays were pulsed with

1 μCi/well of [^{3}H]Thymidine for the last 8 hrs of a 48 hr incubation, harvested on a Filtermate 196 96-well plate harvester (Packard Instrument Co., Meriden, CT), and counted on a Packard TopCount microplate scintillation counter. Results are presented as the mean of triplicate cultures. Standard errors were less than 20% of the mean.

For induction of unresponsiveness, 24 well plates were pre-blocked with 10% FCS supplemented DMEM overnight to prevent soluble anti-CD3-IgG3 from adhering to the plastic. DO 11.10 lymph node cells (2×10^6) or pGL10 clones (1×10^6) were incubated 24 hours in 1 ml media with or without 1-10 μg/ml soluble anti-CD3-IgG3, CsA (1 μg/ml), anti-CD28 (1 μg/ml), $2\text{-}3 \times 10^6$ T-depleted irradiated BALB/c splenocytes, washed three times, and then rested 72 hrs at 37°C. TCR re-expression was verified *via* FACS analysis. For the secondary stimulation, $4\text{-}5 \times 10^4$ DO 11.10 lymph node cells or pGL10 cells were plated in the presence of $2\text{-}5 \times 10^5$ T-depleted (anti-Thy-1 + complement) irradiated splenocytes and 1 μg/ml soluble 145-2C11 or 800 μg/ml OVA. Cultures were pulsed with [^{3}H] Thymidine after 48 hours. For IL-2 production, 2.5×10^4 cells per well were stimulated in a 96 well flat bottom plate with immobilized anti-CD3 plus anti-CD28 at 1 μg/ml. 24 hour supernatants from 3 wells were pooled and analyzed by ELISA (Endogen, Cambridge, MA). For AE.7 assays, 1×10^6 T cells per well were incubated for 24 hrs with 1 μg/ml of anti-CD3-IgG3 mAb, washed, rested, and then in a flat bottom 96 well plate, 4×10^4 T cells were restimulated in the presence of 5×10^5 T-depleted irradiated B10.A splenocytes and 10 μM pigeon cytochrome c.

In other studies described herein below the studies were performed in 5% FCS supplemented DMEM (Life Technologies, Grand Island, NY). In a 96-well flat-bottom dish, 1×10^5 T cells per well were cultured with media alone, 1 μg/ml plate-immobilized anti-CD3 (145-2C11), a single dose of soluble anti-CD3 IgG3 (1 μg/ml), or serial log dilutions of soluble anti-CD3 IgG3. For cytokine-blocking assays, 5×10^4 T cells from the T cell clone 4.5 (or 24.5) were stimulated in the presence of 1 μg/ml of anti-CD3 (anti-CD3 IgG3) with or without APC (2.5×10^5 Thy-1-depleted splenocytes irradiated at 2000 rad), 25 μg/ml of anti-IL-4 mAb, 10 μg/ml cach of anti-IL-2 plus anti-IL-2R mAbs, or 25 μg/ml of rat control Ig

mAb. After 40 h, cultures were pulsed with [^{3}H]TdR for a further 8 h, harvested on a 96-well Filermate 196 plate harvester (Packard Instrument, Meriden, CT), and then counted on a Packard TopCount microplate scintillation counter. Results are represented as the mean of triplicate determinations with a SEM of ≤20%.

Biochemistry. Analysis of anti-CD3 IgG3 mAb-induced TCR phosphorylation has been previously described in detail (Smith *et al.*, 1997). T cell clones or BALB/c lymph node cells were washed in PBS and then resuspended in ice cold PBS at 1×10^8/ml or 2×10^8/ml, respectively. Anti-CD3-IgG3 was added at 4-5 μg/ml for a 10 minute incubation on ice. An equal volume of anti-Ig cross linker or PBS pre-warmed to 37°C was added and samples were incubated a further 2.5-5 minutes in a 37°C water bath. For anti-CD3-Fos and anti-CD3 × anti-CD4 stimulations, cells were stimulated with 10 μg/ml of antibody. After the incubation, an equal volume of ice cold 2× lysis buffer was added (final concentration: 0.5% TritonX, 50 mM Tris (pH 7.6), 100 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 10 μg/ml each Leupeptin and Aprotonin, 25 μM NPGB, and 1 mM PMSF). For immunoprecipitations, 20 μl of a 50% Protein A-agarose bead slurry (Pharmacia-UpJohn, Upsala, Sweden) were coated with 200 μl of mAb-containing supernatant or 2 μl of antisera for one hour at 4°C. Lysates were added to the pre-coated Protein A-agarose beads and incubated one hour at 4°C. The samples were resolved on a 12% SDS polyacrylamide gel for ζ immunoprecipitations or an 8% gel for PLCγ-1, and then transferred to PVDF membrane (Millipore, Bedford, MA). Blots were blocked with 10% bovine serum albumin (Sigma, St Louis, MO) and probed with anti-phosphotyrosine. In some studies, these blots were stripped and reprobed with anti-ZAP-70. For analysis of MAP kinase activation, T cells were stimulated as above, and then 1×10^6 cell equivalents of whole cell lysate was resolved on 10% SDS-PAGE. The blots were probed with anti-active MAP kinase, stripped, and then reprobed with anti-MAP kinase. After incubation with the appropriate horseradish peroxidase-coupled secondary Abs, the blots were developed by enhanced chemiluminescence (Amersham, Arlington Heights, IL). Densitometry measurements of the MAP kinase bands were performed using an AMBIS Image Acquisition and Analysis instrument (San Diego, CA).

Calcium Flux. pGL10 were washed with DMEM containing 10 mM HEPES at pH 7.0 and then incubated at 5×10^6 cells/ml with 5 μM indo-1 at 37°C for 30 minutes. An equal volume of DMEM with HEPES and 5% FCS at pH7.4 was added and cells were incubated 30 minutes. Cells were washed twice with 5% FCS supplemented DMEM at pH 7.2 and resuspended at 1×10^6 /ml for data acquisition on a FACSstar plus (Becton Dickenson Immunocytometry Systems, Mountain View, Ca). Cells were briefly warmed before stimulation with anti-CD3-IgG3 (1 μg/ml) plus rabbit anti-IgG3 (1:30). An increase in the 404/495 nm indo-1 emission ratio is indicative of a rise in intracellular Ca^{2+}. Results were analyzed using Multitime (Phoenix Flow Systems, San Diego, CA).

Confocal Microscopy. Purified DO 11.10 lymph node T cells (10^6) were incubated with 5 μg/ml 145-2C11-FITC on ice for 10 minutes and then stimulated with an equal volume of 37°C pre-warmed goat anti-hamster in PBS (1:300 final) for 0 or 5 minutes at 37°C. T cells were transferred to a polystyrene FACS tube and fixed in 3% paraformaldehyde for 10 minutes at room temperature, washed 3 times with PBS, and then resuspended in 25 μl of mounting solution (0.5 mg/ml O-Phenylenediamine, 90%Glycerol, 0.05 M Tris pH 8.0, 0.2% NaN_3). Samples were analyzed on a ZEISS 410 confocal microscope.

Other studies used 2 to 5×10^6 T cells in serum-free media were stimulated in 100 to 200 μl with 5 to 10 μg/ml anti-CD3 IgG3 for 20 min at 37°C. The cells were then added to an equal volume of 2 to 4% paraformaldehyde for 10 min at room temperature, washed (1% BSA containing PBS), and further permeabilized with −70°C methanol for 2 min on ice. Cells were washed and rehydrated with wash buffer for 15 min at room temperature. Nonspecific staining was blocked with 5% normal goat serum for 20 min at room temperature, and then cells were incubated with a 1:300 to 1:1000 dilution of anti-NF-ATc overnight at 4°C. The cells were washed, incubated for 45 min at 37°C with goat anti-mouse FITC (1:50 final), and then incubated for 15 min with wash buffer at 37°C. After one more wash, the cells were resuspended in Fluoromount-G (Southern Biotech, Birmingham, AL), and mounted on slides for analysis on a Zeiss 410 confocal microscope.

Generation and function of "humanized" anti-CD3 mAbs. Permanent myeloma transfectants of the murine and human-OKT3 mAbs genes were developed as previously described. Mutation of the phenylalanine-leucine sequence at position 234-235 into alanine-alanine to decrease the affinity of the mAb for human and murine FcτRI and II were performed as previously described (Alegre, 1992). ELISAs using a combination of goat anti-human Fc and kappa Abs were performed to determine the yield of assembled "humanized" antibody in COS cell supernatants or permanently transfected myeloma cell-lines (Woodle, 1992).

For T cell proliferation assays, PBMCs, in complete medium (RPMI-1640 plus 10% FCS), were incubated at $1x10^6$ cells/ml (final volume= 200 μl) with serial log dilutions of each antibody in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) for three days at 37 °C. All mAbs samples were airfuged at >30 psi for 20 minutes prior to the assay to remove preformed aggregates (Beckman, Carlsbad, CA). ^{3}H-Thymidine (NEN-DuPont, Wilmington, DE) was added at 1 μCi/well and the plates were incubated for additional 4 hours before harvesting. The cells were harvested in an automatic 96-well cell harvester (Tomtec, Orange, CT) and ^{3}H-thymidine incorporation was measured with a Betaplate Liquid Scintillation Counter (Pharmacia).

Construction and treatment of hu-SPL-SCID mice. Fresh human spleens were obtained from cadaveric organ donors, under a protocol approved by the University of Chicago Institutional Review Board. A single cell suspension was prepared as described. Briefly, 4 to 6 week-old SCID mice were τ-irradiated (200 rad), prior to the intraperitoneal (ip) injection of 10^8 cells/mouse. The percentage of human cells in the peripheral blood was determined by flow cytometry (FCM). First, the peripheral blood mononuclear cells (PBMCs) were incubated (15 minutes) with unlabelled murine IgG antibodies to block subsequent FcτR binding. Next, the cells were stained with PE-coupled anti-murine class I (PharMingen, San Diego, Ca) and counterstained with FITC-coupled anti-human CD45 mAb (Coulter Immunology, Hialeah, FL) to identify the population of human cells. The proportion of human cells is expressed as a percentage of the total number of cells. The

animals bearing between 5 and 20% human cells in the PBMCs were selected for further experiments. Mice, matched for their level of engraftment of human cells in the peripheral blood, received either PBS (1 ml), 145-2C11, OKT3, 209-IgG1 or Ala-Ala-IgG4 (100 μg resuspended in 1 ml of PBS, unless stated otherwise in the text), intraperitoneally (ip) 11 days to 3 weeks after the injection of the human splenocytes.

Detection of circulating anti-CD3 mAbs. SCID and hu-SPL-SCID mice were bled by retroorbital venous puncture 24h, 48h and 1 week after the injection of the mAbs (100 μg ip). The serum titers of the anti-CD3 mAbs were determined by FCM analysis using human PBMNs obtained from EDTA-anticoagulated whole blood of normal volunteers and isolated by Ficoll-Hypaque (Lymphoprep, Nycomed, Oslo, Norway) density gradient centrifugation. Six concentrations of purified OKT3, 209-IgG1 and Ala-Ala-IgG4 in 3-fold dilutions were used to generate standard curves. Human PBMCs were incubated with 3 serial dilutions of each serum (1:10, 1:30 and 1:90), and then stained with FITC-coupled goat anti-mouse Ig (Boehringer-Mannheim, Indianapolis, IN) for detection of OKT3, and with goat anti-human Ig (Caltag Laboratories, San Francisco, CA) for detection of the humanized antibodies. Serum levels were extrapolated from the mean fluorescence of anti-CD3 stained cells, as compared with a corresponding concentration of the purified anti-CD3 mAbs on the standard curves.

Detection of circulating IL-2. Sera obtained from SCID and hu-SPL-SCID mice 2h after anti-CD3 or control treatment were analyzed for the presence of IL-2 was analyzed using a colorimetric assay that utilized the IL-2/IL-4-dependent cell line, CTLL-4, as previously described (Mosmann, 1983). CTLL-4 cells proliferated similarly to recombinant murine and human IL-2, and responded to murine but not human IL-4. To exclude participation of murine cytokines in the proliferation observed, an anti-murine IL-4 mAb, [11B11 (Ohara, 1985)], and an anti-murine IL-2 mAb, [S4B6, (Cherwinski, 1987)], were added to selected wells at concentrations found to block proliferation of CTLL-4 cells to murine IL-4 and IL-2, respectively, but not to human IL-2.

Skin grafting. Neonatal human foreskin was grafted on SCID and hu-SPL-SCID mice 11 days after the inoculation of human splenocytes. Mice were anesthetized with 60 μg/ml of chlorohydrate (120 μl delivered ip) (Sigma, St. Louis, MO) and intermittent inhalation of hydroxyflurane (Metophane, Pitman-Moore, Mundelein, IL). Skin grafts were positioned on the dorsal thorax of the mice. Each foreskin was used to graft 4 animals, each from a different group (SCID, PBS-treated, 145-2C11-treated and anti-CD3-treated hu-SPL-SCID mice). Mice received OKT3, 209-IgG1, Ala-Ala-IgG4 or 145-2C11 (50 μg/day for 5 days, followed by 10 μg/day for 10 days) diluted in 1 ml of PBS, or 1 ml of PBS alone. The grafts were unwrapped at 7 days and the status of the graft was scored blindly and independently by 2 investigators daily for the first 30 days, and once a week afterwards. The scores ranged from 0 to 4: grade 0 represented skin grafts intact and soft; grade 1, skin grafts with a modified pigmentation in a small area; grade 2, soft skin grafts with larger areas of depigmentation; grade 3, those hardened or slightly scabbed; grade 4, shrinking or scabbing skin grafts. Rejection was recorded when scores were grade 3 or greater.

EXAMPLE 2

Specific Examples Of Production Of Humanized Anti-CD3 Monoclonal Antibodies

The present example reports exemplary mutations in murine OKT3 monoclonal antibodies to create a class of "humanized" anti-CD3 monoclonal antibodies. Following the teachings of the present Example, one of skill in the art could make any number of humanized antibodies.

A. Mutation in the Fc portion of the human-OKT3 mAb.

Mutations of the phenylalanine in position 234 into a leucine to increase the affinity of the binding of the mAb to FcR I (Leu-234), or of the contiguous leucine (235) into a glutamic acid to reduce FcR binding (Glu-235) were performed as follows: ultracompetent CJ 236 E. coli (Invitrogen, San Diego, CA) were transformed with pSG5 containing the heavy chain gene of the gOKT3 mAb. The bacteria were allowed to grow in LB broth

supplemented with uridine (25 mg/mL), ampicillin (100μg/mL) until reaching an optical density of 0.35 at a wave length of 600 nm. The CJ 236 E. coli were infected with helper phage M-13 (pfu) (Stratagen) to generate uridine incorporated single stranded template. An oligonucleotide synthesized with thymidine and containing the desired mutation was then annealed to the uridine-single-stranded template to serve as a primer for the replication of the plasmid after the addition of deoxynucleotides, T7 polymerase and T4 ligase; the wild type DNA thus contains uridine, while the mutated plasmid obtained utilizes thymidine. The synthesis reaction was stopped with EDTA 0.5M and Tris HCl-EDTA 1M, and 10 μl were transformed into competent DH5 E. coli that degrade uridine-DNA and thus grew on ampicillin-selected media when transformed with the mutated construct. The plasmid was isolated by Qiagen minipreps; the mutated sequence in pSG5 was co-introduced with the psG5 vector containing the light chain of the mAb into COS-1 cells for transient expression of the mutant immunoglobulin.

B. Generation and identification of OKT3 variable region sequences.

OKT3 variable region sequences were derived from oligo-dT primed cDNA from OKT3 hybridoma cells using the Amersham International Plc. cDNA synthesis kit. The cDNA was cloned in pSP64 using EcoR1 linkers. *E. coli* clones containing light and heavy chain cDNAs were identified by oligonucleotide screening of bacterial colonies using the oligonucleotides: 5' TCCAGATGTTAACTGCTCAC (SEQ ID NO: 15) for the light chain, which is complementary to a sequence in the mouse kappa constant region, and 5' CAGGGGCCAGTGGATGGATAGAC (SEQ ID NO: 16) for the heavy chain, which is complementary to a sequence in the mouse igG2a constant CH1 domain region.

The amino acid sequences for the variable regions deduced from the sequences of the cDNAs are shown in FIG. 1A (row 1) for the light chain and FIG. 1B (row 1) for the heavy chain. The CDR's are shown with the single underlining. The light chain is a member of the mouse V_L subgroup VI and uses a J_K4 minigene. The heavy chain is probably a member of the mouse V_H subgroup II, most probably IIb, although it also has significant homology to the consensus for group Va. The D region is currently unclassified and the J_H region is J_H2. In terms of the loop predictions for the hypervariable regions proposed by Chothia et al., 1987,

the loops can be assigned to canonical structures 1 for L1, 2 for L2 and 1 for L3, and to canonical structures 1 for H1 and 2 for H2, Chothia et al., have not yet predicted canonical forms for H3.

The light chain variable region amino acid sequence shows a high degree of homology to the Ox-1 germline gene and to the published antibodies 45.2.21.1, 14.6b.1 and 26.4.1 (Sikder, 1985). The heavy chain variable region amino acid sequence shows reasonable homology to a subgroup of the J558 family including 14.6b.1. Some antibodies with these combinations of light and heavy chain genes have previously been shown to have affinity for alpha-1-6 dextran.

C. Design and construction of humanized OKT3 genes.

The variable region domains for the humanized antibodies were designed with mouse variable region optimal codon usage (Grantham, 1986) and used the signal sequences of the light and heavy chains of mAb B72.3 (Whittle, 1987). Immediately 5' to the initiator ATG a 9bp Kozak sequence (Kozak, 1987), GCCGCCACC (SEQ ID NO: 17), was inserted. 5' and 3' terminal restriction sites were added so that the variable regions could be attached directly to the DNA sequences for the human IgG4 and Kappa constant regions prior to cloning into the eukaryotic expression vectors.

The variable regions were built either by simultaneously replacing all of the CDR and loop regions by oligonucleotide directed, site-specific mutagenesis (Ollo, 1983) of a previously constructed humanized variable region for B72.3 cloned in M13 or by assembling the sequence using synthetic oligonucleotides ranging in size from 27-67 base pairs and with 6 base overhangs. The oligonucleotides were synthesized on an Applied Biosystems Model 380B DNA Synthesizer and purified by HPLC. The oligonucleotides were enzymatically phosphorylated, paired, annealed and then equimolar aliquots of each pair were mixed and ligated. The cloning sites were exposed by restriction digestion of the ligation mixture and the correctly sized fragments were identified and cloned directly into the expression vectors, 5' to the constant regions, prior to sequencing and expression.

For the design of the humanized OKT3 variable region sequences, REI (Kabat, 1987) was chosen as the human light chain framework, and KOL was chosen for heavy chain variable region. In both cases antibodies were selected for which a structure had been determined by X-ray crystallography so that a structural examination of individual residues in the human variable region frameworks could be made. The variable region sequences of the human acceptor frameworks are shown in FIG. 1A and 1B (row 2) (SEQ ID. NOS: 7 and 11.

For comparison purposes, the amino acid and nucleotide sequences for murine OKT3 (SEQ ID NOS: 2-5 and 1), as obtained from *Sequences of Proteins of Immunbiological Interest* 4/e (Kabat, 1987), are provided in FIG. 2.

Row 3 in each of FIG. 1A (SEQ ID NO: 8) and 1B (SEQ ID NO: 12) shows the sequences for the variable regions of the initial design, gL and gH. Only differences from the human acceptor sequence are shown. For gL the CDR choices were as suggested by Kabat et al., and no other non-CDR murine residues were used. For gH the OKT3 CDR's, as suggested by reference to Kabat et al., were substituted into the KOL sequence along with the murine residues at positions 27, 28 and 30 which are normally bound in a loop region adjacent to CDR1 (Chothia, 1987; 1989). The importance of residue 27 as a determiner of antigen binding was shown by Riechmann et al., (Reichman, 1988) in the reconstitution of binding activity of the CAMPATH-1 antibody. The residues 28 and 30 are predicted to be at the surface of the antibody and near to CDR1. Residue 29 is the same in both KOL and OKT3 (Figure 1B) and therefore does not require to be altered.

The DNA sequences coding for the initial humanized light and heavy variable regions were constructed by simultaneous replacement through site-directed mutagenesis of sequences in previously generated light and heavy chain DNAs of a humanized form of antibody B72.3. The DNA sequences coding for the humanized variable regions were then attached to the human gamma-4 and kappa constant region sequences and inserted into expression vectors as described for the chimeric genes. The gL and gH genes, when co-expressed in COS cells yield antibody gOKT3-1.

gOKT3-1 binds poorly to HPB-ALL cells and is not able to block the binding of mOKT3 to the cells (FIG. 3). Therefore it was clear that further OKT3 residues outside of the CDRs needed to be considered for substitution into the humanized antibody. For the light chain these positions are at 1 and 3 which by reference to known structures for antibody variable regions are probable surface residues located near to the CDR's, residue 46 which is usually at the domain interface and the packing residue at 47, gLA has all four residues derived from the murine sequence while gLC has murine residues at positions 46 and 47 only.

Similarly, for the heavy chain, a number of locations were considered. These were at positions 23, 73 and 76 which are believed, by analogy with known antibody structures, to be partly or completely solvent exposed residues near the CDRs; at positions 6, 24, 48, 49, 71, 78 and 88 which are residues believed either to be involved in positioning of the CDRs and/or in intradomain packing, and the variable domain interface residue 91. Finally at residue 63 in CDR2, which is usually an intra-domain packing residue, the residue found in KOL was used so that potentially unfavorable contacts with other packing residues from the human framework could be avoided. A number of light and heavy chain variants were built to assess the contribution of these framework residues. It was found by experiment that residues 1 and 3 on the light chain were not required to be derived from the murine sequence, but that one or both of residues 46 and 47 should be derived from the murine sequence. FIG. 1A, row 4 (SEQ ID NO: 9) shows the sequence of gLC which differs from gL by having the murine sequences at residues 46 and 47. Similarly, in the heavy chain it was found that while incorporating all of the modifications described above to give gHA (FIG. 1B row 4) (SEQ ID NO: 13), and co-expressing this gene with cL or gLC would lead to antigen binding equivalent to cOKT3 or mOKT3, some of the residues were not necessary to retain equivalent binding affinity. In particular it was found when the KOL sequences were used at positions 71, 73, 76, 88 and 91 in the gHG gene, co-expression of gHG with cL or gLC led to antigen binding equivalent to cOKT3 or mOKT3. Therefore, the binding affinity of the gLC/gHA(gOKT3-5) and gLC/gHG(gLC/gHG) combinations have been analyzed in more detail.

Large scale COS cell expression preparations were made and the humanized antibody was affinity purified by Protein A. Relative binding affinities were measured. FIG. 3 shows results from two such experiments. The affinity of mOKT3 for antigen (K_a) was measured to be 1.2 x 10^9 M^{-1} by Scatchard analysis. This value for mOKT3 compares well to that of 1.3 x 10^9 M^{-1} by Scatchard analysis. This value for mOKT3 compares well to that of 1.3 x 10^9 M^{-1} determined previously (Gergely, 1990). In FIG. 3A, gOKTE-5 was compared with cOKT3 and mOKT3 for competition against mOKT3. Values of 1.2 x 10^9 M^{-1} and 1.1 x 10^9 M^{-1} 2343 obtained for the cOKT3 and gOKT3-5 antibodies respectively.

Subsequently, (FIG. 3B) similar results were obtained for gOKT3-7 (K_a 1.4 x 10^9 M^{-1}) compared to 1.2 x 10^9 M^{-1} for mOKT3, 1.4 x 10^9 M^{-1} for cOKT3 and 1.1 x 10^9 M^{-1} for gOKT3-5. These experiments show that the antigen binding activity of OKT3 has been successfully transferred to the humanized antibodies.

Previous studies have indicated that mitogenic potency is a sensitive parameter of the T cell activation properties of anti-CD3 mAbs (Woodle, 1991). In an earlier study it was shown that gOKT3-5 still demonstrated mitogenic potency even in the context of an IgG4 isotype. Therefore, the activation potency of gOKT3-7 antibody was assessed by quantitating proliferating responses. gOKTE-7 demonstrated mitogenic potency equivalent to that of mOKT3 (FIG. 4). This suggests that cross-linking of the bound antibody still occurs with the γ4 isotype leading to proliferative signals. A therapeutic humanized OKT3 antibody may need further alterations to the constant region to minimize such effects.

D. Construction and expression of chimeric OKT3 genes.

The murine cDNAs were assembled into expression vector controls for the biological function of the humanized antibodies. The murine variable region cDNA sequences were attached to human k light chain and γ4 heavy chain constant region DNA sequences following a previously described strategy to generate chimeric OKT3 (cOKT3) genes which were then inserted into eukaryotic expression vectors. As the ultimate aim is to design a humanized OKT3 iGg antibody which can efficiently bind to CD3 while retaining useful

effector pharmacokinetics and have no first dose side effects, a reduced affinity for FcR was built into the constructs by using the γ4 gene.

Small scale COS cell expression and metabolic labelling studies were as described (Whittle, 1987). Large scale COS cell expression studies were performed in roller bottles, harvesting the product supernatant 5 days after transfection. (T. Livelli, Specialty Media Inc., Lavallette, New Jersey). Material from large scale transfections was purified by Protein A Sepharose chromatography. The yield of assembled antibody in COS cell supernatants was measured as described by Woodle et al., 1992.

Murine OKT3, cOKT3, and murine/chimeric hybrid antibodies expressed from COS cells were shown to bind to antigen equivalently to mOKT3 and to block the binding of MOKT3 to CD3 positive cells.

E. Transient expression of murine and human-OKT3 mAbs genes.

COS-1 cell expression studies were performed using reagents and procedures from a transient expression kit (Specialty media, Lavallette, NJ) modified for use in roller bottles (T. Livelli, Specialty Media, personal communication). Product supernatants for purification of the test Abs were harvested 6 days after transfection.

ELISA assays were performed to determine the yield of assembled "humanized" antibody in COS cells supernatants. Ninety-six well plates were coated with $F(ab')_2$ goat anti-human Fc antibody. COS cell supernatants were added and incubated for one hour at room temperature and washed. Horseradish peroxidase-conjugated goat anti-human kappa chain (Caltag) was used with o-phenylenediamine (OPD) for detection. Purified human IgG was used as standard.

F. Mutated "humanized" OKT3 mAbs bind to the CD3 complex of T cells with the same affinity as murine OKT3.

The Fc portion of the gOKT3-5 mAb was mutated according to procedures described above in order to alter its binding to FcR-bearing cells. A phenylalanine was substituted for a

leucine in position 234 (Leu-234), or the adjacent leucine (235) was transformed into a glutamic acid (Glu-235). The affinity of the gOKT3-5 mAb for the TCR complex was previously shown to be similar to that of OKT3 (Van Wauwe, *et al.*, 1980). Although changes in the Fc portion of the mAb should not alter Ag binding affinity, it was important to show that point mutations in the CH2 region of the Ab, close to the hinge, did not impair the binding of the Leu-234 and the Glu-235 mAbs to the CD3 antigen.

A displacement assay was performed to examine the ability of the mutated Abs to competitively inhibit the binding of murine OKT3 to human T cells. Human peripheral blood acute lymphocytic leukemia cells were re-suspended in flow cytofluorimetry (FCM) buffer at 5 x 10^5 cells/mL. Dilutions of the anti-CD3 mAbs were added and incubated at 4°C for 1 hour. Fluorescein isothiocyanate (FITC) was dissolved in N,N-dimethyl formamide (DMF) to give a 10 mg/ml solution. FITC/DMF was added to purified mAb at 1:10 w/w and incubated at 25°C for four hours, followed by dialysis into PBS containing an anion exchange resin (AG1-X8, 200-400 mesh, chloride form; Bio-Rad). Aggregates were removed prior to use by airfuge centrifugation (Becton-Dickinson). A fixed saturating amount of OKT3-FITC was added, and the cells were further incubated for 1 hour at 4°C, washed and analyzed by flow cytofluorimetry (FCM).

One or two-color FCM were performed using a FACScan flow cytometer, interfaced to a Hewlett-Packard 310 computer. Data analysis were performed using Consort-30 software. Logarithmically amplified fluorescence data were collected on 10,000 viable cells, as determined by forward and right angle light scatter intensity. One-color fluorescence data were displayed in histogram mode with fluorescence intensity on the *x* axis and cell number of the *y* axis. Two-color fluorescence data were displayed as contour plots with green (FITC) fluorescence on the *x* axis and orange (phycoerythrin) fluorescence on the *y* axis. All FCM staining procedures were performed at 4°C in FCM buffer.

The results of this assay are shown in FIG. 5. The data is presented as % inhibition of maximal fluorescence intensity (determined by OKT3-FITC binding in the absence of blocking Ab). Both mutant Abs displayed a similar affinity for their epitope as the parental

gOKT3-5 mAb. In contrast, the gOKT3-6 mAb, a different "humanized" OKT3 which has a very weak binding activity for the CD3 antigen (Van Wauwe, *et al.*, 1980), was unable to displace the OKT3 mAb. These results correlate with the data obtained previously on a panel of isotype-switch variants of murine anti-CD3 mAbs. In those studies, the anti-CD3 mAbs expressing different isotypes had a comparable avidity for the TCR complex as assessed by Scatchard analysis (Van Wauwe, *et al.*, 1980), or by precipitation of the TCR complex and cross-blocking experiments. Thus, any differences in the activation or suppressive properties of the mutated Abs could not be attributed to a modified affinity of the combining site of the anti-CD3 mAbs for T cells.

G. Binding of the mutant anti-CD3 mAbs to FcR on U937 cells.

The mutations generated in the CH2 region of the human IgG4 gOKT3-5 either mimicked the amino acid sequence of the FcR binding region of a human IgG1 (Leu-234), which has a higher affinity for human FcR I than human IgG4, or of a murine IgG2b (Glu-235) that binds weakly to FcR I but still binds to human FcR II. In order to determine the effects of those mutations on FcR binding, the FcR binding affinity of the various "humanized" OKT3 mAbs were tested on the monocytic U937 cell line that bears FcR I and II by displacement of either a PE-coupled murine IgG2a or of a ^{125}I-labelled human IgG1.

The murine anti-CD5 IgG2a-PE, OKT3E IgG2b, OKT3D IgG2b, OKT3 IgG2a, and a human IgG4 Ab FITC-coupled as described *supra*, were used to compete for binding in the FcR binding assay. Phycoerythrin-coupled (PE) anti-CD2 and anti-CD5 used as counterstains in the activation assays were purchased from Coulter Immunology. Modulation and coating of the TCR were determined using FITC-coupled OKT3 IgG2a and OKT3D IgG2a as described below.

FcR binding assays were performed using the FcR I- and II-bearing U937 human cell line.

For competitive inhibition assay with PE-coupled murine anti-CD5 IgG2a, 30 x 10^6 cells were cultured overnight at 37°C in complete media in the presence of 500 U/mL of

human IFN-γ to enhance the expression of FcR I. The cells were washed three times with DMEM containing 25 μM HEPES, incubated for 2 hours at 37°C in FCS-free media and washed twice in DMEM and once in flow cytofluorimetry (FCM) buffer (PBS containing 0.1% FCS and 0.1% sodium-azide). Aliquots of the anti-CD3 mAbs serially diluted in FCM buffer, were added to 96 well V-bottom tissue culture plates along with 250,000 U937 cells/well. After incubating the cells for 15 mins. at 0°C, 0.3μg of anti-CD5 was added. Displacement of Fc-mediated anti-CD3 binding was allowed to occur for 90 minutes at 0°C, after which cells were harvested and washed in FCM buffer. Fluorescence of 10,000 cells stained with the PE-anti-CD5 Ab was determined using a FACScan flow cytometer. The data was plotted in a format using Consort 30 software as described below.

For competitive inhibition assay for FcR binding with ^{125}I-human IgG, U937 cells were washed and re-suspended at a concentration of 1.4×10^8 cells/mL in the assay medium (0.2% BSA in PBS). Aliquots of 1×10^6 cells per tube were incubated for 1h at 37°C with ^{125}I-labeled human IgG at a final concentration of 1×10^{-9} M. Murine or "humanized" OKT3 was added at final concentrations ranging from 0.023 μg/ml to 150 μg/mL, with the total volume equaling 21 μL/tube. Following the incubation, the mixture was layered over 10% sucrose. Upon centrifugation at 11000 g for 5 mins, the pelleted cells (bound ^{125}I-huIgG) separated from the medium containing free ^{125}I-huIgG. The tubes were then frozen in dry ice and the bottom of the tube containing the pelleted cells was removed for analysis of the bound ^{125}I-huIgG.

The maximum binding of ^{125}I-huIgG was determined in the absence of the inhibitor. The results are expressed as a percentage of the ^{125}I-huIgG bound in the presence of the inhibitor relative to the maximum binding. Non-specific binding is seen as the percentage bound in the presence of excess inhibitor (150 μg/ml murine OKT3). All controls and samples were assayed in triplicate tubes.

The N-terminal of the CH_2 domain of the mutated constructs is summarized in FIG. 6.

Murine OKT3 IgG2a had the highest affinity of all the anti-CD3 mAbs tested for FcR on U937 cells. As previously shown for human IgG4 mAbs, the gOKT3-5 required a 10-fold higher concentration to achieve the same inhibition. The Leu-234 mAb, that was expected to enhance FcR binding, has consistently proven to compete more efficiency for FcR binding than the gOKT3-5 mAb. In contrast, the Glu-235 mAb, bearing the FcR binding region similar to murine IgG2b, bound poorly to U937 cells, requiring a 10-fold higher concentration than the gOKT3-5 and approximately a 100-fold greater concentration than the murine OKT3 to achieve the same percent inhibition. These results indicated that, as anticipated from their respective amino acid sequence in the FcR binding domain, the rank order of binding of the mAbs to U937 cells was murine OKT3>Leu-324>gOKT3-5>Glu-235 mAb.

H. Proliferation Assays.

The Glu-235 mAb was tested for its ability to induce T cell proliferation. Human peripheral blood mononuclear cells (PBMC) were obtained from normal volunteers by Ficoll-hypaque density gradient centrifugation of EDTA-anticoagulated whole blood. EBV-transformed lymphoblastoid cell lines (LCL) and human histiocytoma-derived U937 cell-line were maintained in continuous culture in complete media (DMEM supplemented with 2mM L-glutamine), 2 mM non-essential amino acids, 100 U/mL penicillin-streptomycin (Gibco), $5x10^5$ M 2-mercapto-ethanol (Gibco) and 25 μM HEPES (Gibco) with 10% fetal calf serum (FCS, Gibco).

PBMC preparations were re-suspended in complete DMEM with 1% FCS and aliquotted to 96-well round bottom tissue culture plates (Costar) at $1x10^5$ cells/well. The different Abs were added to the wells by serial log dilutions in culture media. After 72 hours of culture at 37°C in a 5% CO_2 incubator, 1 μCi of ^{3}H-thymidine was added to each well and followed by an additional 24 hour incubation. Cells were harvested on a semi-automatic cell harvester and ^{3}H-thymidine incorporation was measured in a liquid scintillation counter. All data were expressed as mean CPM of triplicate determinations.

Stimulation of PBMC with the wild-type gOKT3-5 mAb resulted in cell proliferation comparable to that observed with PBMC stimulated with murine OKT3, as shown in FIG. 7. In contrast, no proliferation was induced by the Glu-235 mAb using PBMC from 3 different donors at mAb concentrations up to 10 μg/ml, suggesting that the alteration of the FcR binding region of this mAb had impaired its mitogenic properties.

I. Activation of T cells by CDR-grafted mutant mAbs.

In order to further analyze early T cell activation events, human peripheral blood mononuclear cells (PBMC), cultured with various anti-CD3 mAbs, were assessed for cell surface expression of Leu 23 and IL-2 receptor at 12 and 36 hours incubation, respectively.

For studies involving T cell expression of activation markers, 2 x 10^6 PBMC were cultured for either 12 hours (Leu 23 expression) or 36 hours (IL-2 receptor expression) in 24 well tissue culture plates in the presence of varying concentrations of the mAbs.

No significant differences were reproducibly observed between murine OKT3 and gOKT3-5 mAb with respect to expression of these cell surface markers (*see* FIG. 8). In contrast, activation by the Glu-235 mAb resulted in lower levels of expression of both markers. In fact, the highest concentration of the Ab used (10μg/mL) achieved less than 40% of the maximal activation obtained with standard OKT3. No differences in the expression of these markers were observed between $CD4^+$ and $CD8^+$ cells.

J. IFN-g, GM-CSF and TNF-a production induced by "humanized" OKT3 mAbs.

The acute toxicity observed in transplant recipients after the first administration of OKT3 has been attributed to the systematic release of lymphokines triggered by the mAb. Therefore, the *in vitro* production of GM-CSF, TNF-α and IFN-γ induced by the "humanized" anti-CD3 mAbs was measured. For studies involving lymphokine production, 2 x 10^6 PBMC were cultured in 24-well plates for either 24 hours (TNF-α) or 72 hours (GM-CSF and IFN-γ). Tissue culture supernatants were collected at the completion of the

respective incubation periods and stored at -20°C. Lymphokine levels were measured via sandwich ELISA techniques using commercially available kits.

Similar amounts of cytokines were produced after culture of PBMC with OKT3 and gOKT3-5 mAb. In contrast, the highest concentration of the Glu-235 mAb induced small quantities of TNF-α (*see* FIG. 9) and GM-CSF, and no IFN-γ.

K. Induction of modulation and coating of the TCR complex by molecularly engineered OKT3 mAbs.

The immunosuppressive properties of the different mAbs was compared *in vitro*. First, the mAbs were examined for their capacity to modulate and/or coat the TCR complex. Human peripheral blood mononuclear cells (PBMC) were incubated at $1x10^6$ cells/mL for 12 hours in 24 well plates with known concentrations of anti-CD3 mAb. PBMC from each group were harvested and stained with either OKT3-FITC or OKT3D-FITC. The fluorescein-stained cells were counterstained with anti-CD5-PE to identify T lymphocytes and analyzed by flow cytofluorimetry (FCM). OKT3D-FITC was selected because of its binding to an epitope distinct from the one binding OKT3 mAb. Thus, this Ab provided a direct measurement of unmodulated surface CD3.

Formulae for calculating CD3 coating and modulation were:

$$\%\ \text{CD3 Mod.} = \frac{\text{Control Cells } MC_{OKT3D\text{-}FITC} - \text{Ab-treated cells } MC_{OKT3D\text{-}FITC}}{\text{Control Cells } MC_{OKT3D\text{-}FITC}}$$

$$\%\text{CD3 Coated} = \left(\frac{\text{Ab-treated Cells } MC_{OKT3D\text{-}FITC}}{\text{Control Cells } MC_{OKT3D\text{-}FITC}} - \frac{\text{Ab-treated Cells } MC_{OKT3\text{-}FITC}}{\text{Control Cells } MC_{OKT3D\text{-}FITC}}\right) x100$$

% CD3 Uncoated + Unmodulated = 100 (% CD3 Coated + % CD3 Modulation)

where MC represents the mean channel along the x-axis.

As shown in FIG. 10, the combined modulation and coating of the TCR complex achieved by the gOKT3-5 and murine OKT3 were very similar, with half-maximal TCR blocking achieved at approximately 1 ng/ml. However, the half-maximum modulation plus coating observed with the Glu-235 mAb required a 100-fold greater concentrations of mAb (1 μg/mL) than of murine OKT3. The major difference between the Glu-235 mAb and the other Abs was due to a change in kinetics since, by 48 hours, the mAb coated and modulated the TCR complex similarly to OKT3. Thus, the achievement by Glu-235 mAb of internalization of the TCR, which may depend on multivalent cross-linking, was delayed as compared with the other anti-CD3 mAbs.

L. Inhibition of CTL activity by CDR-grafted mutant mAbs.

The ability of the Abs to suppress cytoxicity of alloreactive T cells was compared. HLA-A2-specific CTL were generated from a normal HLA-Al donor. Cytolytic activity was assessed on FcR negative-EBV-transformed HLA-A2 target cells. CTL were generated by a bulk allogeneic MLC technique. Normal human donors were phenotyped for HLA-A expression. Responder and stimulator combinations were selected specifically to generate HLA-A2-specific CTL effectors. Responder and stimulator PBMC were prepared by Ficoll-hypaque density gradient centrifugation as described above and re-suspended in RPMI 1640 with 2mM L-glutamine, 100 U/mL penicillin-streptomycin, 25 μM HEPES and 15%. decomplemented normal human serum. Stimulator PBMC (1 x 10^7/mL) were irradiated (3000 rad) and cultured with responder PBMC (1 x 10^7/10mL) in upright 25 cm tissue culture flasks. After 7 days of culture, freshly irradiated stimulator PBMC (4 x 10^6/10mL) were added to 4 x 10^6/10mL of the initial cultured cells and incubated for an additional five days. Cells were then harvested and assayed for CTL activity by ^{51}Cr release.

HLA-A2-specific CTL effectors were generated as described above, harvested and aliquotted to a 96 well U-bottom tissue culture plate at four different effector/target ratios. Effectors were pre-incubated with serial dilutions of each anti-CD3 mAb for 30 minutes. Following incubation with mAbs, ^{51}Cr-labeled Fc receptor negative-target cells [HLA-A2 expressing LCL line (Z2B) or HLA-A1 expressing LCL line (G12B) used as a non-specific target] were added. Spontaneous lysis was measured by incubation of targets alone in media

and maximal lysis was achieved by addition of 0.05 N HCL. Effectors and targets were co-cultured; supernatant aliquots were harvested and radioactivity was measured in a gamma-counter.

T cell cytotoxicity was specific as demonstrated by the absence of lysis of a syngeneic HLA-A1 EBV-transformed cell-line. Inhibition of lysis by anti-CD3 mAbs previously has been attributed to the inability of the T cells to recognize their targets, due to TCR blockade by the mAb. In the present study, murine OKT3, gOKT3-5 mAb and Glu-235 exhibited a comparable inhibitory effect on the cytolytic activity of the alloreactive T cells. These results suggest that the ability of the different mAbs to coat the TCR within the 30 min incubation time was similar (*see* FIG. 11). In contrast, the gOKT3-6 mAb, a "humanized" OKT3 that has a significantly reduced binding activity for the CD3 antigen, did not inhibit CTL activity. These results suggest that modified affinities for FcRs do not alter the immunosuppressive property of the anti-CD3 mAbs, *in vitro*.

Results were calculated using the following formulae:

$$\% \text{ Specific lysis} = \frac{\text{Experimental CPM - Spontaneous CPM}}{\text{Maximal CPM - Spontaneous CPM}}$$

$$\% \text{ Maximal specific lysis} = \frac{\% \text{ Specific lysis}_{[mAb]}}{\% \text{ Specific lysis}_{Control}}$$

Where % Specific $\text{lysis}_{[mAb]}$ represents the CPM obtained at a given mAb concentration for a E:T ratio of 25:1 and % Specific $\text{lysis}_{Control}$ represents the CPM obtained in the absence of mAb at the same E:T ratio. Results were expressed as the mean of triplicates.

M. CD4 modulation studies.

PBMCs isolated from Ficoll-Hypaque density gradient centrifugation were incubated at 1 x 10^6 cell/mL with known concentrations of OKT3 antibodies at 37° C for 24 hours. The cells were harvested and stained with FITC-OKT4. The cells were counterstained with PE-

labelled anti-CD5 (PE-Leu1, Becton Dickinson Immunocytometry Systems, San Jose, CA) to distinguish T lymphocytes from other PBMCs, and analyzed by FACScan. Data from the resulting studies are reported in FIG. 1 (Transy, 1989).

%CD4 modulation was calculated as follows:

$$\frac{\text{Control } MCN_{FITC\text{-}OKT4} - \text{Ab treated } MCN_{FITC\text{-}OKT4}}{\text{Control } MCN_{FITC\text{-}OKT4}} \times 100$$

The data in the left plot of FIG. 12 reveal that the humanized antibodies studied induce the modulation of CD4 in a dose-dependent manner. In contrast is the data for mOKT3 (solid circles), the antibody from which the humanized and mutated antibodies were constructed, had no effect on CD4, as indicated by a straight line plot between antibody concentrations of from 0.01 to 0.10 μg/mL. The same can be said for the mOKT3D IgG2b antibody (solid triangles) which has also been neither humanized nor mutated.

The right plot indicates that, as expected, there is no modulation of CD8 for any of the antibodies studied.

N. ELISA and RES-KW3 studies of CD4 binding.

RES-KW3 cells were washed with PBS+0.2%BSA+0.1% sodium azide (staining buffer), and first incubated with various concentrations of OKT3 antibodies for 1 hour on ice. The cells were washed three times with cold staining buffer, and FITC-labelled goat anti-human or goat anti-mouse antibodies were added (Caltac Lab. So. San Francisco, CA). The cells were incubated on ice for another hour before being washed and subject to FCM.

FCM was performed using a FACScan (Becton-Dickinson Immunocytometry Systems, Mountain View, CA) flow cytometer interfaced to a Hewlett-Packard 340 computer, data analyzed using lysis II software (Becton Dickinson). Fluorescence data were collected using logarithmic amplification on 10,000 viable cells as determined by forward and right

angle light scatter intensity. One-color fluorescence data were displayed in histogram mode with fluorescence intensity on the x axis and relative cell number on the y axis.

HIVgp120/CD4 receptor EIA coated microplates from DuPont were used in the CD4 binding assay. 100 μL/well of CDR-grafted OKT4AIgG1 at various concentrations (1:2 dilution at starting concentration of 50 ng/mL) was added into the wells duplicate for the construction of standard curve. 100 μL/well of OKT3 antibody samples at various dilutions wee then added. The diluent is PBS+10% calf serum+0.05% Tween-20. The plates were incubated at room temperature for 2 hours.

The plates were washed with PBS+0.05% Tween-20 six times before 100 μL/well of 1:15000 diluted HRPO-conjugated goat anti-human x(f+B) antibodies in diluent was added. The plates were incubated at room temperature for another 2 hours. The plates were washed six times again, and 100 μL/well of the OPD/hydrogen peroxide solution (five 2-mg OPD tablets were added in 13 mL of Mili-Q water; after they were dissolved, 5 μL of 30% hydrogen peroxide were then added) was added into each well. The plates were incubated at room temperature in the dark for 30 minutes, and 50 μL/well of 2.5N HCl was added to stop the reaction. The plates were then read at 490 nm.

The resulting data are reported in FIG. 13 and 15. These data indicate that the humanized OKT3 binds to CD4, either immobilized to ELISA plates or bound to the surface of RES-KW3 cells.

O. Generation of a Non-Activating Anti-CD3 mAb Based on gOKT3-7.

To generate an anti-human CD3 mAb with an improved therapeutic index, the inventors have developed a panel of "humanized" anti-CD3 mAbs derived from OKT3, by molecularly transferring the complementary determining regions (CDRs) of OKT3 onto human IgG1 and IgG4 molecules (Woodle *et al.*, 1992; Adair *et al.*, submitted for publication). In addition, the inventors examined whether immunosuppression can be achieved by anti-CD3 mAbs in the absence of the initial step of cellular activation. The "humanized" mAb, formally named gOKT3- 7(τ_1), abbreviated 209-IgG1, that has a high

affinity for human FcτRs was shown, in vitro, to have similar activating properties to OKT3 (Alegre, 1992) and would therefore be expected to induce in patients the acute toxicity associated with lymphokine release by activated T cells and FcτR-bearing cells. A second mAb, formally named gOKT3-7(τ_4-a/a); abbreviated Ala-Ala-IgG4, was developed with 2 amino acid substitutions in the CH_2 portion (from a phenylalanine-leucine to an alanine-alanine at positions 234-235) of the "humanized" gOKT3-7(τ4) (209-IgG4) mAb. These mutations significantly reduced binding of the mAb to human and murine FcτRI and II and led to markedly reduced activating characteristics *in vitro* (Alegre, 1992). Importantly, this variant mAb retained the capacity to induce TCR modulation and to prevent cytolysis *in vitro*, and thus represents a potential new immunosuppressive therapeutic agent.

Severe combined immunodeficient (SCID) mice carry an autosomal recessive, spontaneously arising mutation that results in the inability to successfully rearrange immunoglobulin and TCRs. These animals are therefore devoid of T and B lymphocytes (McCune, *Annu. Rev. Immun.*, 1991; McCune, *Curr. Opin. Immun.*, 1991; Bosma, 1983; Bosma, 1991). The inventors have recently developed a model in which lightly irradiated SCID mice are injected with human splenocytes from cadaveric organ donors. These hu-SPL-SCID mice maintain functional human T cells capable of responding to mitogens and alloantigens *in vitro*, and of acutely rejecting human foreskin allografts *in vivo*. In the present study, the inventors have utilized hu-SPL-SCID mice to assess the immunosuppressive properties of the non-activating "humanized" anti-CD3 mAbs *in vivo*.

P. Results

a. Characteristics of the "humanized" mAbs.

OKT3 and the "humanized" mAbs were shown in companion studies to have similar avidities for the human CD3 complex, as determined by flow cytometry (FCM) in a competitive binding assay using FITC-coupled OKT3 (Alegre, 1992). In a competitive inhibition assay for FcR binding using ^{125}I-human IgG and the human monocytic cell-line U937, OKT3, 209-IgG4 and 209-IgG1 were found to have similar affinities for human FcτRs, whereas the binding of the Ala-Ala-IgG4 and Ala-Ala-IgG1 mAbs to human FcτRI or FcτRII were greatly reduced. Finally, the "humanized" mAbs were tested for their ability to

induce T cell proliferation. Stimulation of PBMCs with the 209-IgG4 or 209-IgG1 mAbs resulted in cell proliferation comparable to that observed with PBMCs stimulated with murine OKT3 (FIG. 16). In contrast, no significant proliferation was induced by the Ala-Ala-IgG4 mAb at concentrations up to 100 ng/ml. In fact, the proliferation observed at the highest concentrations may be due to aggregation of the mAb. These results suggest that the alteration of the FcτR-binding region of this mAb had impaired its mitogenic properties.

b. Determination of the circulating levels of anti-CD3 mAbs.

Ten days to three weeks after injection of 108 human splenic cells in the peritoneal cavity, SCID mice were tested for the percentage of human cells engrafting their peripheral blood. As previously described, graft versus host disease (GVHD) was apparent in mice bearing more than 25 to 30% human cells. Therefore, in order to minimize the level of human T cell activation prior to anti-CD3 treatment, animals with 5% to 20% circulating human $CD45^+$ cells were selected for subsequent experiments. Mice matched for their level of engraftment with human cells were assigned to different groups for treatment with OKT3, 209-IgG1, Ala-Ala-IgG4 or PBS. As shown in Figure 17, significant serum levels of all of the anti-CD3 mAbs (between 8 and 13 μg/ml) were measured 24h after the injections. No anti-CD3 mAb was detected in SCID or hu-SPL-SCID mice treated with PBS. The persistence of the mAbs was relatively short, inasmuch as levels decreased dramatically by 48h. These data are consistent with results reported previously of a short half-life of immunoglobulins in other hu-SPL-SCID experimental models (Duchosal, 1992). They also are reminiscent of the time course for clearance of circulating OKT3 following its injection into humans (Thistlethwaite, 1988).

c. Depletion of T cells following administration of anti-CD3 mAbs.

The injection of OKT3 and 209-IgG1 into hu-SPL-SCID mice induced a rapid and substantial depletion of circulating human $CD45^+$ cells, that was almost maximal when first measured, 3h after the injection. These data are consistent with the clearance of T cells from the peripheral blood seen in humans following the injection of OKT3. Interestingly, the depletion observed in the peripheral blood after administration of Ala-Ala-IgG4 in hu-SPL-SCID mice was consistently less striking than after the injection of the activating

anti-CD3 mAbs, suggesting that binding of the anti-CD3 mAbs to FcτRs might play a role in the reduction of the number of circulating T cells. The clearance of human cells from the spleen and peritoneal cavity was not complete after a single injection of any of the anti-CD3 mAbs, activating or non-activating. In addition, the kinetics of depletion in the spleen were slower than in the peripheral blood, with maximal loss of 60% of the human cells not achieved until 48h. In contrast, a protocol analogous to that employed clinically in human transplant recipients, consisting of 14 consecutive days of i.p. administration of the anti-CD3 mAbs (10 μg), resulted in a complete depletion of $CD3^+$ T cells in the peripheral blood, the spleen and the peritoneal cavity even after Ala-Ala-IgG4. This absence of $CD3^+$ cells was not due to modulation and/or coating of the TCR complex by mAbs, inasmuch as staining with PE-coupled anti-CD4 or anti-CD8 mAbs did not reveal any remaining human T cells. Furthermore, hu-SPL-SCID splenocytes harvested 3 days after the completion of this protocol were unable to proliferate to immobilized OKT3, *in vitro*. It is interesting to note that the ability of OKT3 to deplete T cells from human lymphoid compartments such as spleen or lymph nodes is unknown. However, studies using the anti- mouse CD3 mAb, 145-2C11, have shown that T cells are also depleted from the peripheral lymphoid organs of the immunocompetent mice.

d. Induction of surface markers of activation on T cells after administration of anti-CD3 mAbs.

An early event following injection of OKT3 into transplant recipients is the activation of $CD3^+$ T cells due to the cross-linking of the TCR by $FcτR^+$ cells (Abramowicz, 1989; Chatenoud, 1989; Ceuppens, 1985). T cell activation in patients results in increased surface expression of markers such as CD69, CD25 and HLA-DR. As previously described, a significant percentage of hu-SPL-SCID T cells express CD25 and HLA-DR, as a result of GVHD. In contrast, levels of CD69, which is an earlier and more transient marker of activation, are comparable to those found on T cells from humans. A significant increase in the expression of $CD69^+$ on both $CD4^+$ and $CD8^+$ splenocytes was observed 24h after the injection of OKT3 and 209-IgG1 into hu-SPL-SCID mice, but not after the administration of Ala-Ala-IgG4 or PBS (Figure 18), suggesting that the Ala-Ala-IgG4 mAb induced less T cell activation than the FcτR-binding anti-CD3 mAbs.

e. Production of IL-2 after anti-CD3 therapy.

The administration of OKT3 to patients has been shown to induce the rapid systemic release of cytokines such as TNF-α, IL-2, IL-6 and IFN-τ, peaking 2 to 6h after the injection (Abramowicz, 1989; Chatenoud, 1989). This cytokine production results in the acute toxicity associated with anti-CD3 therapy in transplant recipients. In the present study, a bioassay was used to measure the serum level of human IL-2 2h after treatment of hu-SPL-SCID mice with PBS, OKT3, 209-IgG1, Ala-Ala-IgG4 or 145-2C11, a hamster anti-murine CD3 mAb. As shown in Figure 19, only the injection of OKT3 and 209-IgG1 induced the release of detectable human IL-2 in hu-SPL-SCID mice. The levels detected were low because of the relatively small percentage of engrafted human cells, but readily detectable in the experiments performed. The lymphokine production from individual animals varied as a consequence of the different percentage of human cells engrafting each animal. No human or murine IL-2 was detected after injection of 145-2C11, confirming the absence of endogenous murine T cells in these mice. The administration of Ala-Ala-IgG4 did not induce IL-2 production, consistent with the reduced ability of this mAb to fully activate human T cells. To verify the human origin of the cytokines detected, polymerase chain reaction assays were performed on spleens of SCID and hu-SPL-SCID mice 6h after treatment, using primers that did not cross-react with murine cytokines. In addition to IL-2, IFN-τ mRNA was found to be up-regulated after injection of the OKT3 and 209-IgG1 mAbs, but not the Ala-Ala IgG4 mAb. Together, these results demonstrate that the Ala-Ala-IgG4 mAb has reduced activating properties as compared with OKT3 and 209-IgG1.

f. Prolongation of skin graft survival by the administration of anti-CD3 mAbs.

The immunosuppressive properties of the different mAbs was next examined. Previous studies have shown that the 209-IgG1 and the Ala-Ala-IgG4 mAbs were both effective at modulating TCR and suppressing cytotoxic T cell responses *in vitro* (Alegre, 1992). Initial studies *in vivo* suggested a similar rapid immunosuppressive effect induced by both "humanized" mAbs, as TCR was significantly modulated from the cell surface 24h following injection of either mAb. However, in order to directly explore the

immunosuppressive efficacy of these mAbs, the inventors performed skin graft experiments. Previous studies from the inventors' laboratory have shown that hu-SPL-SCID mice are capable of rejecting human foreskin allografts and that human T cells participate in this process. SCID and hu-SPL-SCID mice were grafted with human foreskin obtained from circumcisions and assumed to be allogeneic with respect to the human cells used for the adoptive transfer. Hu-SPL-SCID mice matched for their level of human CD45 expression in the peripheral blood received either PBS or daily doses of OKT3, 209-IgG1, Ala-Ala-IgG4, or 145-2C11 for 15 consecutive days, beginning on the day of the skin graft. As shown in Figure 20, animals that received PBS or 145-2C11 rejected their grafts with a 50% mean survival time of 13 days, consistent with the inventors previous results. In contrast, all of the OKT3- treated animals and all but 1 of the 209-IgG1- and Ala-Ala-IgG4-treated mice maintained their skin grafts for greater than 80 days. Mice were sacrificed at 80 days, and 2 animals per group were analyzed for the percent of human cells in the different cellular compartments. None of the anti-human CD3-treated mice reexpressed human $CD3^+$ cells in the peripheral blood, the spleen or the peritoneal cavity, as determined by FCM. In contrast, the PBS-treated animals retained a significant percentage of human $CD45^+$ and $CD3^+$ cells in the different compartments although the absolute numbers were reduced over time, as compared with the initial engraftment. Three additional skin graft experiments have been performed with 5-7 animals per group. In these experiments, 66-80% of the animals treated with OKT3, 209-IgG1 and Ala-Ala-IgG4 maintained their grafts for as long as the animals were examined. In two of the three experiments, a higher percentage of mice treated with the Ala-Ala-IgG4 maintained their skin grafts permanently. No statistical difference was found between these 3 groups.

Q. DISCUSSION

These studies suggest that a "humanized" mAb derived from OKT3 and bearing mutations of 2 amino acids in the Fc portion to impede its binding to FcτRs does not induce human T cell activation *in vivo* in a preclinical model, but retains the immunosuppressive properties of the native mAb.

OKT3 has been shown to mediate T cell activation by cross-linking T lymphocytes and FcτR$^+$ cells (Palacios, 1985; Ceuppens, 1985). Because hu-SPL-SCID mice are chimeric animals comprising both murine and human FcR$^+$ cells, it was important to use mAbs that would have similar avidities for human and murine FcτRs. Thus, OKT3, a murine IgG2a, and the human 209-IgG1 mAb have a high affinity for FcτRs of both species. In contrast, the human Ala-Ala-IgG4 bears mutations dramatically reducing its binding to murine and human FcτRs. The efficacy of engraftment of the different cellular compartments with human B cells, monocytes/macrophages and NK cells, as providers of human FcτR, is relatively low in this hu-SPL-SCID model [10% in the peritoneal cavity and the peripheral blood and 20% in the spleen, when compared to the proportion of human T lymphocytes observed. On the other hand, murine monocytes/macrophages and NK cells are functionally normal in SCID mice and express normal levels of murine FcτR (Bosma, 1991; Kumar, 1989). The type of accessory cell responsible for the cross-linking mediated by OKT3 and 209-IgG1 in this chimeric system, whether murine or human, was adequate to trigger cellular activation analogous to that observed in patients after the injection of OKT3. Indeed, OKT3 and 209-IgG1-triggered activation of the human T lymphocytes was evident in the treated mice, as determined by the production of human IL-2 and the accumulation of human IFN-τ mRNA, as well as by the increased expression of the surface marker of activation, CD69, on T cells. In contrast, the inability of Ala-Ala-IgG4 to interact with FcτRs rendered this mAb incapable of fully triggering T cell activation.

The activation of T lymphocytes and FcτR$^+$ cells in patients treated with OKT3 is associated with adverse reactions such as fever, chills, headaches, acute tubular necrosis, diarrhea, acute respiratory distress syndrome etc. (Abramowicz, 1989; Chatenoud, 1989; Toussaint, 1989; Thistlethwaite, 1988; Goldman, 1990). Similarly, immunocompetent mice injected with 145-2C11 develop hypothermia, hypoglycemia, lethargy, liver steatosis and acute tubular necrosis (Alegre, *Eur. J. Immun.*, 1990; Alegre, *Transplantation*, 1991; Feran, 1990). Hu-SPL-SCID mice did not exhibit detectable symptoms after OKT3 or 209-IgG1 therapy if the percentage of human cell engraftment was moderate. However, when animals with more than 30% human cells in their PBMCs were injected with OKT3 or 209-IgG1, they became extremely lethargic and an increased percentage of animal deaths was observed. As

shown previously, animals engrafted with a high percentage of human T cells often undergo a GVHD-like syndrome, that results in a number of pathological symptoms including pancreatitis, diffuse hemorrhagic necrosis and in many instances animal death. Interestingly, the administration of Ala-Ala-IgG4 to highly engrafted animals seemed to reduce the symptoms of GVHD and perhaps even prevent some deaths. The number of animals examined was, however, too small to generate statistical differences.

The administration of all 3 anti-CD3 mAbs to hu-SPL-SCID mice, whether activating or not, resulted in modulation of the CD3 molecules from the surface of T lymphocytes and subsequent T cell depletion. Similarly, in transplanted patients treated with OKT3, rapid modulation of the TCR complex and T cell depletion from the peripheral circulation are presumably responsible for the immunosuppressive properties of the drug (Chatenoud, 1982). Importantly, in this study, the administration of the Ala-Ala-IgG4 mAb resulted in dramatic prolongation of allograft survival similarly to the activating OKT3 and 209-IgG1 mAbs. These findings indicate that complete T cell activation due to T lymphocyte/FcR^+ cell cross-linking may not be necessary for the achievement of a potent anti-CD3-mediated immunosuppression.

In summary, the Ala-Ala-IgG4, a mAb bearing 2 amino acid mutations in the Fc portion of a "humanized" OKT3, may prove useful in clinical transplantation to induce immunosuppression while being less immunogenic and induce less adverse reactions than OKT3. In addition, the use of a "humanized" mAb may lessen the generation of anti-xenotypic Abs that often arise after repeated administrations of OKT3 (Thistlethwaite, 1988). Finally, the non-activating Ala-Ala-IgG4 mAb might also widen the applications of anti-CD3 mAbs to patients suffering from autoimmune diseases, in whom treatment with OKT3 was never realized because of the potential adverse reactions and the strong humoral responses induced by the mAb.

EXAMPLE 3

An Anti-CD3-IgG3 Monoclonal Antibody Is Fc Receptor Non-Binding Due To Insufficient Cross-Linking Of The TCR.

Unlike the original 145-2C11 mAb, the anti-CD3-IgG3 chimeric antibody does not induce proliferation or IL-2 production in whole spleen cells (Alegre *et al.*, 1995). Also, soluble 145-2C11 failed to induce proliferation of T cell clones in the absence of FcR-mediated cross-linking. To directly test the role of multivalent cross-linking, a secondary IgG3-specific cross-linking antibody was added to cultures containing the anti-CD3-IgG3 mAb. The addition of the cross-linking reagent reconstituted a mitogenic stimulus for both fresh murine splenocytes and a T cell clone (FIG. 20). Thus the induction of proliferation by anti-CD3 requires a higher order of TCR aggregation that cannot be achieved by bivalent Ab binding alone.

EXAMPLE 4

Fc Receptor Non-Binding Anti-CD3 Renders T Cell Clones Hyporesponsive.

Although insufficient for induction of T cell proliferation or cytokine production, the anti-CD3-IgG3 mAb may deliver at least a "partial" signal which alters T cell function. Therefore, the effects of anti-CD3-IgG3 on the functional responses of naive cells and Th1 clones were examined. pGL10 T cells or DO.11.10 lymph node cells were cultured in the presence of splenic accessory cells (to compensate for the presence of non-T cells in the naive population) and Fc receptor non-binding anti-CD3-IgG3. Previous studies have shown that treatment of T cells with anti-CD3-IgG3 resulted in down-modulation of TCR expression within 24 hours (Alegre, 1993). Therefore, after 24 hrs., the cells were washed, and recultured for 3 days to allow re-expression of the TCR. As seen in FIG. 21A, upon restimulation with the mitogenic 145-2C11 mAb plus splenic APCs, thymidine incorporation by anti-CD3-IgG3 treated pGL10 was markedly reduced as compared to pGL10 cultured with media alone. In contrast, the functional responses of murine lymph node T cells were not affected by culture with anti-CD3-IgG3. The clonal unresponsiveness did not merely reflect decreased viability, since anti-CD3-IgG3 treated clones proliferated in the presence of exogenously added IL-2. The effect of anti-CD3-IgG3 was not specific to the pGL10 clone

since the Fc receptor non-binding anti-CD3 rendered the pigeon cytochrome C specific clone, AE.7, hyporesponsive as well (FIG. 21B). To determine whether the reduced proliferation of anti-CD3-IgG3 treated T cell clones correlated with IL-2 production, pGL10 clones were cultured with or without anti-CD3-IgG3 for 24 hrs, rested, and then restimulated with immobilized anti-CD3 plus anti-CD28 (PV-1), conditions known to induce readily detectable IL-2 production (FIG. 21C). Anti-CD3-IgG3 treated clones secreted significantly less IL-2 than the media treated control cells. These data indicated that exposure to soluble, non-cross linked anti-CD3 selectively reduces the responsiveness of Th1 clones as compared to naive cells.

To examine whether the presence of CsA or CD28 costimulation would affect the Fc receptor non-binding anti-CD3 induced unresponsiveness, pGL10 T cells were cultured with Fc receptor non-binding anti-CD3 alone, or Fc receptor non-binding anti-CD3 in the presence of CsA, or splenic APCs and anti-CD28 (FIG. 21D). CsA partially blocked the induction of unresponsiveness by Fc receptor non-binding anti-CD3, suggesting that this process may depend upon a calcium signal. In contrast, addition of anti-CD28 mAb in the primary culture failed to restore secondary responses.

EXAMPLE 5

Fc Receptor Non-Binding Anti-CD3 Delivers A Partial TCR Signal.

The functional consequences of culture with Fc receptor non-binding anti-CD3 support the hypothesis that anti-CD3-IgG3 delivers a signal. Therefore studies were performed to determine the nature of the TCR signal triggered by Fc receptor non-binding anti-CD3. Upon ligation of the TCR, one of the earliest events to occur is the tyrosine phosphorylation of components of the TCR complex (ζ and CD3 ε,δ, and γ) (Qian *et al.*, 1993). Phosphorylation of these chains allows subsequent association and phosphorylation of a variety of other proteins, including the protein tyrosine kinase, ZAP-70 (Weiss and Littman, 1994). T cells were stimulated with the anti-CD3-IgG3 mAb in the presence or absence of a secondary Ig cross-linker. The TCR complex was immunoprecipitated with anti-ζ and analyzed for tyrosine phosphorylation. Stimulating T cells with anti-CD3 under cross-linking

conditions induced both 21 kd and 23 kd forms of phosphorylated ζ (p21 and p23) as well as phosphorylation of CD3 ε. The phosphorylated band below p21 (~18kd) most likely represents another isoform of phosphorylated ζ (Reis e Sousa *et al.*, 1996). In contrast, the non-cross-linked anti-CD3-IgG3 mAb induced similar levels of phosphorylated CD3 ε and p21 ζ, but significantly less p23 ζ. Quantitation of the p21 and p23 bands by densitometry in multiple T cell clone studies (n = 4) revealed a consistent correlation between the degree of anti-CD3 cross-linking and the p23/p21 ratio; conditions that promote cross-linking increased the relative level of p23 expression (FIG. 22).

Examination of the phosphoproteins which co-precipitated with the ζ chain in darker exposures or greater cell number revealed further differences between anti-Ig cross-linked and non cross-linked conditions. Unlike the cross-linked anti-CD3 stimulation, several of these phosphoproteins (bands between 30 and 46 kd as well as at 70 kd and 76 kd) were missing or reduced in the anti-ζ precipitations from T cells stimulated with the non-cross linked anti-CD3 mAb. The proximal signals triggered by Fc receptor non-binding anti-CD3 in lymph node T cells were similar to those induced in clones in that a) Fc receptor non-binding anti-CD3 induces phosphorylation of TCR chains and b) in the absence of crosslinking, several TCR associated phosphotyrosine containing proteins are missing or reduced in intensity. These results suggest that although Fc receptor non-binding anti-CD3 induces some tyrosine phosphorylation of ζ and the CD3 chains, it is deficient in triggering other proximal signaling events.

Previous studies have shown that the 70 kD band observed in anti-ζ precipitates represents the TCR-associated tyrosine kinase, ZAP-70 (Chan *et al.*, 1991). The reduced intensity of this band in the immunoprecipitates from Fc receptor non-binding anti-CD3 treated cells could either represent a failure of ZAP-70 association or deficient phosphorylation. In order to investigate whether stimulation with non-cross-linked anti-CD3 is sufficient for TCR association, 2×10^7 pGL10 T cells were stimulated with PBS, goat anti-IgG3 alone, anti-CD3-IgG3 or anti-CD3-IgG3 plus anti-IgG, for 2.5 minutes at 37°C, lysed, and immunoprecipitated with anti-ζ. Blots were probed with anti-ZAP70, and then stripped and reprobed with anti-phosphotyrosine. The non-cross-linked anti-CD3-IgG3

induced similar levels of ZAP-70 recruitment to the TCR complex; yet as confirmed by re-probing the blot with anti-phosphotyrosine, the proportion of ZAP-70 which was tyrosine phosphorylated was significantly reduced. Thus, in the absence of CD3 cross-linking, ZAP-70 associates with the TCR/CD3 complex, but it is not efficiently phosphorylated.

EXAMPLE 6

Defects In Downstream Events In The Absence Of TCR/CD3 Cross-Linking

The differences in proximal signal transduction observed in the absence of cross-linking were likely to be reflected in critical downstream biochemical events, such as PLCγ-1 activation. pGL10 were stimulated with anti-CD3-IgG3 in the presence or absence of cross-linker (2×10^7 pGL10 cells were stimulated for 5 minutes at 37°C as indicated. Samples were precipitated with anti PLCγ-1, and then resolved on an 8% gel. The western blot was probed with anti-phosphotyrosine, stripped, and then reprobed with anti-PLCγ-1). The dramatic increase in PLCγ-1 phosphorylation observed in the presence of a secondary cross-linking Ab was not observed following anti-CD3-IgG3 stimulation alone. Similarly, cross linking with anti-IgG enhanced PLCγ-1 tyrosine phosphorylation induced by the Fc receptor non-binding anti-CD3 in naive cells (4×10^7 lymph node cells were stimulated for 5 minutes with PBS, anti-CD3-IgG3 or anti-CD3-IgG3 plus goat anti-IgG3 and analyzed as above).

Since anti-CD3-IgG3 was unable to induce significant PLCγ-1 phosphorylation, it was anticipated that one of the events which depends upon PLCγ-1 activation, Ca^{++} mobilization, would likewise be impaired. T cell clones were loaded with the calcium sensitive dye indo-1 and then analyzed by FACS for calcium flux. A calcium flux was not detected when the cells were stimulated with the anti-CD3-IgG3 alone, even after 5 minutes. However, in T cells incubated with anti-CD3-IgG3 followed by the addition of a secondary cross-linker, a characteristic calcium flux was observed within one minute (FIG. 23). Anti-Ig Abs in the absence of anti-CD3 did not result in a calcium flux . These results demonstrate that the downstream signaling events of PLCγ-1 activation and the ensuing Ca^{2+} flux are dependent upon extensive cross-linking of the TCR/CD3 by anti-CD3 mAbs.

Aggregation of the TCR complex has been shown to correlate with T cell activation; Kupfer *et al.* demonstrated that when T cells encounter antigen/MHC on APCs, the TCR redistributes on the cell surface to form an aggregated "activation cap" (Kupfer *et al.*, 1987). This redistribution is a signaling dependent process most likely involving reorganization of the cytoskeleton (Selliah *et al.*, 1996; Rozdzial *et al.*, 1995; Valitutti *et al.*, 1995). To test whether the addition of a cross-linking Ab to anti-CD3 results in an aggregated TCR cap, confocal microscopy was performed on pGL10 and purified DO.11.10 T cells incubated with anti-CD3 (2C11-FITC) under cross-linking vs. non-cross-linking conditions. In the presence of cross-linking Ab, anti-CD3 stimulation induces aggregation of the TCR into a cap on one side of the cell. However, in the absence of cross-linker, the anti-CD3 remained diffusely distributed on the cell surface. Thus the signal delivered by Fc receptor non-binding anti-CD3 appeared insufficient for the redistribution of TCRs into an aggregated cap.

EXAMPLE 7

Recruitment Of CD4/lck Into The Complex Reconstitutes Complete Proximal Signal Transduction And Mitogenicity

The inability of Fc receptor non-binding anti-CD3 to trigger specific downstream events and proliferation most likely stem from the defective proximal events observed involving ζ and ZAP-70. Previous studies have suggested that the src family kinase, lck, plays a crucial role in the phosphorylation of ζ which subsequently allows association and phosphorylation of ZAP-70 (Iwashima *et al.*, 1994; Straus *et al.*, 1996). Thus, it was possible that the differences in ζ and ZAP-70 phosphorylation seen upon the addition of cross-linker to anti-CD3 may have reflected increased lck activation or enhanced recruitment to the TCR. Initial studies examining lck activation by monitoring lck tyrosine phosphorylation revealed no differences between cross-linking and non-cross-linking conditions. It is clear that CD4 associates with lck and can interact with the TCR complex inducibly upon TCR ligation of antigen/MHC. Thus, artificially bringing CD4/lck into the TCR complex might reconstitute a mitogenic anti-CD3 stimulus even in the absence of a secondary cross-linking Ab. In order to test this hypothesis, the inventors took advantage of a bispecific anti-CD3 × anti-CD4 reagent prepared by a molecular approach to insure the presence of monovalent arms specific for

CD3 and CD4 (as described in Materials and Methods). T cells were incubated with anti-CD3-Fos or the anti-CD3 × anti-CD4 bispecific $F(ab)'_2$, lysed and the TCR/CD3 complex was then immunoprecipitated and analyzed. The bispecific construct induced significant p23 ζ, ZAP-70 phosphorylation, as well as association of the phosphoproteins between 30-46 kd even in the absence of a secondary cross linking antibody. In contrast, the overall pattern induced by anti-CD3-Fos resembled the results seen in T cells stimulated with the anti-CD3-IgG3 mAb: specifically, a reduced association of phosphoproteins and barely detectable ZAP-70 phosphorylation. In the lysates of T cells stimulated with the bispecific anti-CD3 × anti-CD4 construct, a large tyrosine phosphorylated protein was observed which migrated just above the heavy chain. This phosphoprotein is likely to be p56 lck based on protein size. This band never appeared in the cross linked anti-CD3 studies. One possible explanation for this difference is that in the absence of CD4 co-aggregation, lck may dissociate from the TCR complex after lck phosphorylates its substrates. Whereas under stimulation conditions using the bispecific antibody, lck remains in the complex longer due to stable association with coaggregated CD4.

The biochemical results suggested that the anti-CD3 × anti-CD4 bispecific antibody was delivering a competent activating signal to the T cells. In fact, T cell clones or fresh murine T cells cultured in the presence of anti-CD3 × anti-CD4 proliferated, whereas T cells cultured with the anti-CD3-Fos did not (FIG. 24A and FIG. 24B). Thus, enhanced association of lck with the TCR complex reconstituted both early signaling events and a mitogenic stimulus in the absence of further Ab cross-linking.

EXAMPLE 8

Anti-CD3 Mabs Inactivate Th1 And/Or IL2 Producing T Cells While Promoting Th2 Type T Cells

In proliferation assays, there was a selective activation of Th2 type cells and no activation of Th1 clones as seen in the proliferative response to immobilized vs. soluble anti-CD-3 (FIG. 25A and FIG. 25B). Non clonal activated T cells produce IL-4 but not IL-2 in response to 2C11-IgG3 (FIG. 26A and FIG. 26B), showing that there is a selective induction

of IL-4 and not IL-2 in bulk activated T-cells. In further assays it was demonstrated that Th2 clones produce IL-4 in the secondary stimulation (FIG. 27). In Restimulation studies with antigen it was demonstrated that FcR non-binding anti-CD3 monoclonal antibodies induce anergy in Th1 but not Th2 clones (FIG. 28A and FIG. 28B).

Table 2

Induction of IL-4 by IgG-3

Cell Type	Stimulation	IL-4 (ng/ml)
pL104	imm.2CU	182
	2CU γ3-3	37.5
PL104	imm.2CU	310
	2CU γ3-3	24
Bulk act. a	imm 2CU	180
	2CU γ3-3	18
Bulk act. b	imm 2CU	95
	2CU γ3-3	6.1

EXAMPLE 9

FCR-Nonbinding Anti-Cd3 Mabs Induce Proliferation And IL-4 Production In Th0 And Th2 Cells

The ability to trigger or suppress different activated Th populations may contribute to the *in vivo* efficacy of FcR-nonbinding anti-CD3. Therefore, the effect of the anti-CD3 IgG3 mAb on Th1 and Th2 responses was compared (FIG. 29A). As previously shown, Th1 T cell clones did not proliferate in response to the soluble bivalent anti-CD3 mAb. However, multivalent cross-linking provided by a secondary anti-IgG Ab (Smith *et al.*, 1997), or immobilization of the anti-CD3 mAb on a plastic surface resulted in proliferation. By comparison, the Th2 clone, pL104, incorporated [^{3}H]TdR in the absence of exogenous mAb cross-linking. In the presence of splenic APC, anti-CD3 IgG3 also promoted clonal expansion of the Th2 culture supernatants revealed that the soluble anti-CD3 IgG3 mAb induced production of the autocrine growth factor IL-4, although the amount produced was consistently less than that observed in response to immobilized anti-CD3 mAbs (FIG. 29B).

Activated T cells designated as "Th0" make both IL-2 and IL-4 before commitment to a Th lineage. The responses of two OVA peptide-specific Th0 clones (4.5 and 24.5) to the anti-CD3 IgG3 mAb were examined. Both Th0 clones proliferated to soluble and immobilized anti-CD3 mAbs (FIG. 30A). As evidence of their Th0 phenotype, the T cell clones produced IL-2 and IL-4 upon culture with immobilized anti-CD3 mAb; however, in response to the anti-CD3 IgG3 mAb, the Th0 clones secreted only IL-4 (FIG. 30B). It is possible that the IL-2 was undetectable due to consumption by the proliferating cells. If true, the Th0 clones would have preferentially consumed IL-2 vs IL-4 when cultured in the absence of anti-CD3 cross-linking. However, anti-IL-4 mAb, but not anti-IL-2/IL-2R mAbs, blocked anti-CD3 IgG3-induced proliferation in Th0 clones (FIG. 31).

In an *in vivo* setting, the presence of costimulating-bearing APC might alter the cytokine profile induced by anti-CD3 IgG3 in Th0 cells. To address this possibility, splenic accessory cells were included in the *in vitro* stimulations (FIG. 31). Despite the presence of APC, Th0 clones produced IL-4 and depended upon IL-4 for proliferation in response to the anti-CD3 IgG3 mAb. In contrast, both anti-IL-4 and anti-IL-2/IL-2R mAbs partially blocked FcR-binding anti-CD3 mAb-induced proliferation. Similar results were obtained with the Th0 clone 24.5. Thus, IL-4 appears to be the preferred growth factor produced in response to the anti-CD3 IgG3 mAb.

One caveat in using T cell clones to predict the behavior of activated T cells is that clones have been restimulated many times *in vitro* and thus selected for long-term survival in tissue culture. During the course of passage, clonal responses could potentially deviate from what might be observed with "normal" activated T cells. Thus, bulk T cells from the DO 11.10 TCR transgenic were activated with Ag and APC *in vitro* one to three times, then challenged with the anti-CD3 IgG3 mAb. At the time of analysis, these polyclonal activated T cells were capable of producing IL-2, IL-4, and IFN-γ. Previously activated DO 11.10 T cells proliferated in response to the soluble anti-CD3 IgG3, in contrast to the lack of response seen in naive T cells (FIG. 32). The T cells stimulated with soluble anti-CD3 IgG3 produced

IL-4, and not IL-2, even though (as seen in response to immobilized anti-CD3) the T cells were capable of producing both cytokines.

To better define which cells were proliferating in response to the anti-CD3 IgG3 mAb within the polyclonal activated T cell population, IL-4KO and IFN-γ KO mice were used to generate Th1 and Th2 populations, respectively. After one round of *in vitro* activation with mitogenic anti-CD3 (2C11) and APC, T cells from the IL-4KO mice produced IFN-γ whereas T cells from the IFN-γKO mice produced IL-4. When challenged with the anti-CD3 IgG3, the activated IFN-γKO T cells proliferated to both soluble and immobilized anti-CD3. In contrast, the activated IL-4KO cells proliferated to immobilized, but not soluble anti-CD3 IgG3 (FIG. 33). Thus, anti-CD3 IgG3 induced proliferation only in the Th2-like, IL-4-secreting populations.

EXAMPLE 10

FCR-Nonbinding Anti-CD3 Induces Unresponsiveness In Th1 And Th0 Cells, But Not Th2 T Cells

The preceding studies suggested that enhanced outgrowth of IL-4-producing cells following FcR-nonbinding anti-CD3 treatment may contribute to the Th cytokine deviation observed in several *in vivo* models. These alterations in Th phenotype could also reflect the selective induction of Th1 unresponsiveness. To examine this latter possibility, the effect of anti-CD3 pretreatment on Th1 vs Th2 clonal responsiveness was determined. T cells were cultured for 24 h with anti-CD3 IgG3, washed extensively, rested for 3 days and then restimulated with optimal doses of Ag and APC (FIG. 34). This 3-day rest period was sufficient for TCR reexpression (Alegre *et al.*, 1995; Smith *et al.*, 1997). Preculturing the Th1 clone, pGL10, with anti-CD3 IgG3 resulted in proliferative hyporesponsiveness that correlated with reduced IL-2 production (Smith *et al.*, 1997). The addition of costimulation-bearing splenic APC did not affect the ability of anti-CD3 IgG3 to induce unresponsiveness in Th1 clones (Smith *et al.*, 1997). In contrast to the Th1 clone, preculture of the Th2 clone pL104 with anti-CD3 IgG3 did not affect the ability of the T cells to respond to Ag, or produce IL-4 in the restimulation assay (FIG. 34A). Next, Th0 clones were examined to

determine the effect of anti-CD3 IgG3 treatment on the ability of dual cytokine-producing T cells to respond in subsequent stimulations. As seen in FIG. 34B, Th0 clones precultured with the soluble anti-CD3 IgG3 were hyporesponsive in a secondary antigenic stimulation (20% of control proliferation). The anti-CD3 IgG3-treated Th0 clones produced readily detectable IL-4 (40% of control), similar to what has been observed in other anergy systems (Gajewski *et al.*, 1994).

Finally, the consequences of anti-CD3 IgG3 pretreatment of bulk Ag-activated T cells were examined (FIG. 35). Culture with the anti-CD3 IgG3 mAb had a minimal effect on T cell proliferation in the secondary antigenic challenge in the majority of studies. In some studies, treatment with anti-CD3 IgG3 following two to three rounds of *in vitro* stimulation resulted in a significant decrease (up to 67%) in proliferative response to Ag. This variation in the effect of anti-CD3 IgG3 on proliferative responsiveness may reflect the relative mixture of Th0, Th1, and Th2 cells that developed during the repeated antigenic stimulations. However, in all the studies, anti-CD3 IgG3 treatment induced a profound deviation in the cytokine profile evident upon restimulation with Ag T cells exposed to anti-CD3 IgG3 produced equal or slightly greater IL-4, and significantly less IL-2 compared with controls.

The observed alteration in the cytokine profile reflected potential contributions from selective outgrowth of IL-4-producing cells as well as Th1 unresponsiveness. However, it was not clear from previous studies whether *in vitro*-activated nonclonal Th1 cells could be rendered anergic. The effects of anti-CD3 IgG3 treatment on the responsiveness of activated T cells derived from the IL-4KO and IFN-γKO mice were compared. Consistent with the observations in T cell clones, anti-CD3 IgG3 induced hyporesponsiveness in IL-4KO but not in IFN-γKO T cells (FIG. 36). Taken together, the results from clonal and polyclonal populations suggest that selective Th hyporesponsiveness may contribute to the cytokine profile changes induced by anti-CD3 IgG3 mAb treatment.

EXAMPLE 11

Th1 And Th2 Clones Show Similar Proximal Signaling Defects In Response To Anti-CD3 IgG3

Studies with T cell clones and polyclonal activated populations indicated that anti-CD3 IgG3 could induce proliferation only in cells capable of producing IL-4. Thus, either the anti-CD3 IgG3 delivers biochemically distinct TCR signals to Th1 and Th2 cells or anti-CD3 IgG3 delivers a similar TCR signal with different outcomes. It had been demonstrated previously that triggering of the TCR on Th1 clones by non-cross-linked anti-CD3 IgG3 resulted in partial phosphorylation of ζ and inefficient phosphorylation of TCR-associated ZAP-70. This proximal signal resulted in downstream decreased in PLCγ-1 activation. For Th1 cells, this perturbation of tyrosine phosphorylation correlated with a tolerogenic signal (Smith *et al.*, 1997).

To address whether anti-CD3 IgG3 delivers more "complete" signal to responder Th2 cell types, proximal signaling events in pGL10 (Th1) or pL104 (Th2) cells were compared. After stimulation with anti-CD3 IgG3 in the presence or absence of an anti-IgG cross-linking reagent, the TCR complex was immunoprecipitated with anti-ζ and the resulting blot probed with anti-phosphotyrosine Abs. Portions of the anti-ζ immunoprecipitations were probed with anti-ζ Abs to confirm that an equivalent amount of TCR complex was present in the different samples. In both Th subsets, similar qualitative differences were observed between cross-linked and non-cross-linked anti-CD3 signaling. Non-cross-linked anti-CD3 IgG3 mAb induced less of the hyperphosphorylated p23 ζ vs p21 ζ, and less ZAP-70 phosphorylation. In Th2 cells, phosphorylated CD3ε and p18 ζ were diminished as well. Examination of aliquots (10% of volume) by Western blotting with an anti-ζ antiserum demonstrated comparable amounts of ζ in each preparation. Probing the TCR blots with anti-ZAP-70 revealed that even in the apparent absence of ZAP-70 phosphorylation in Th2 clones, ZAP-70 was physically associated with the TCR complex. Thus, at the level of the TCR, anti-CD3 does not appear to induce Th2 proliferation by delivering a more complete TCR signal.

EXAMPLE 12

Anti-CD3 IgG3 Induces Defective Downstream Signals In Both Th1 And Th2 Clones

It was possible that even though the proximal anti-CD3 IgG3 signals were defective, the minimal phosphorylation observed was sufficient to induce more complete downstream events in Th2 cells. TCR-induced *ras* activity has been shown to be essential for T cell activation. *Ras* triggers the activation of a series of serine/threonine kinases leading to MAP kinase phosphorylation, activation, and translocation into the nucleus. This signaling cascade culminates in the activation of a composite transcription factor, AP-1, which binds multiple cytokine promoters (Cantrell, 1996). Therefore, MAP kinase phosphorylation was evaluated as an indicator of *ras* pathway induction in anti-CD3 IgG3-triggered T cell responses. In the presence of an anti-IgG3 cross-linker, anti-CD3 IgG3 induced significant MAP kinase phosphorylation. By comparison, the non-cross-linked anti-CD3 IgG3 resulted in much weaker MAP kinase phosphorylation (fourfold less for ERK2 and sevenfold less for ERK1). A functional assay for activation was consistent with MAPK phosphorylation (p44/42 MAP). This reduced phosphorylation in the absence of anti-CD3 cross-linking was not merely due to delayed kinetics. Significantly, *ras* pathway signaling was compromised to the same extent in both Th1 and Th2 clones following anti-CD3 IgG3 stimulation.

Upon ligation of the TCR, a second lipid-mediated pathway is activated as well. A key player in this pathway, PLCγ-1, cleaves phosphoinositol bisphosphate to yield diacylglycerol and IP_3. IP_3 triggers a $Ca^{2\leftarrow}$ flux, which ultimately leads to nuclear translocation of NF-AT (the cytoplasmic portion of the nuclear factor of activated T cells) (Weiss and Littman, 1994). Both NF-ATp and NF-ATc family members have been shown to translocate upon activation (Timmerman *et al.*, 1996). NF-AT is a critical transcription factor for several cytokine genes, including IL-2 and IL-4 (Rao, 1994). In previous studies, an anti-CD3 IgG3-induced calcium flux was not detectable by FACS in Th1 cells (Smith *et al.*, 1997). however, there were several indirect indications suggesting that anti-CD3 IgG3 might induce a subtle calcium signal. The ability of cyclosporin A to block anti-CD3 IgG3-induced Th1 anergy implied that anti-CD3 IgG3 delivered a calcium signal that might be required for

the tolerogenic activity of the mAb (Smith *et al.*, 1997). Furthermore, anti-CD3 IgG3 delivered a sufficient Ca^{2+} signal to synergize with PMA in causing IL-2 production and proliferation in both naive T cells and Th1 clones. Finally, in an APL model, where IP_3 generation had been historically undetectable, extremely sensitive video imaging revealed transient low amplitude calcium fluxes (Sloan-Lancaster *et al.*, 1996). Based on these results, it was likely that anti-CD3 IgG3 delivers a weak calcium signal that might affect NF-AT translocation into the nucleus. Therefore, the translocation of NF-ATc was examined in Th1 and Th2 clones stimulated with anti-CD3 IgG3. The stimulated cells were fixed, stained with anti-NF-ATc, and analyzed by confocal microscopy. Treatment with anti-CD3 IgG3 induced a shift in NF-ATc localization from the cytoplasm (evident as a thin ring) to more diffuse central areas containing bright spots. Thus, in spite of the proximal deficits in TCR signaling manifested in reduced PLCγ-1 and MAP kinase activation, anti-CD3 IgG3 delivered a sufficient signal to induce NF-ATc translocation into the nucleus (Smith *et al.*, 1997).

Discussion

In this study, the inventors have shown that an anti-CD3 mAb with low FcR affinity, anti-CD3 IgG3, delivers a characteristic partial signal with different functional consequences depending upon the Th phenotype of the population. Anti-CD3 IgG3 treatment of mixed activated populations resulted in a relative decrease in the ability of these populations to produce IL-2, without diminishing IL-4 production, recapitulating the findings from *in vivo* studies of anti-CD3 $F(ab')_2$ treatment (Hughes *et al.*, 1994).

The ability of anti-CD3 IgG3 to clonally expand Th2 (IL-4-secreting) cells while suppressing the responsiveness of IL-2-secreting cells provides a mechanism for the Ab-induced Th cytokine deviation evident *in vitro* and *in vivo*. Specifically, anti-CD3 IgG3 induced proliferation in populations of activated T cells capable of producing the IL-4 growth factor. Unlike Th2 cells, IL-2-secreting populations, such as Th1 clones, Th0 clones, and Th1 lines, were rendered hyporesponsive following treatment with anti-CD3 IgG3. For Th0 clones, the reduced responsiveness most likely resulted from the combined lack of IL-2 production and the blockade of IL-4 responsiveness previously reported in other anergy models (Gajewski *et al.*, 1994; Mueller *et al.*, 1991). The contrasting effects of anti-CD3

IgG3 on Th0 and Th2 responsiveness suggests that the induction of unresponsiveness does not strictly correlate with proliferation during the primary culture.

The biochemical signals triggered by anti-CD3 IgG3 mAbs in Th1 and Th2 cells were qualitatively similar. In both T cell subsets, stimulation with the non-cross-linked anti-CD3 IgG3 resulted in a reduced ratio of hyperphosphorylated p23 ζ compared with p21 ζ and minimal ZAP-70 phosphorylation. These proximal deficits were exaggerated in Th2 clones, possibly due to the decreased overall level of tyrosine phosphorylation seen when T cells were stimulated with either cross-linked or non-cross-linked anti-CD3. The quantitative differences may reflect clonal variation, since such differences have been observed among Th1 clones. Similar proximal signaling defects have been demonstrated in the APL system and under conditions of CD4 coreceptor blockade (Sloan-Lancaster *et al.*, 1994; Madrenas *et al.*, 1995; Madrenas *et al.*, 1997). Previous reports in these two model systems have stressed the correlation between these specific signaling deficits and the induction of unresponsiveness in T cell clones. However, the results presented here using a variety of T cell clones and short-term T cell lines provide evidence that an altered ratio of p23 to p21 ζ, and defective ZAP-70 phosphorylation, do not always lead to the induction of unresponsiveness. Rather, the consequences of the proximal signaling defects induced by anti-CD3 IgG3 varied, depending upon the Th phenotype.

In this study, two major TCR signaling cascades, involving the PLCγ-1 and *ras* pathways, were evaluated by examining events proximal to the nucleus. *Ras* activates a series of serine/threonine kinases, ultimately resulting in phosphorylation (and thus activation) of the MAP kinases ERK1 and ERK2 (Cantrell, 1996). In Th1 and Th2 cells, non-cross-linked anti-CD3 IgG3 induced weak phosphorylation of the MAP kinases compared with the cross-linked mAb, indicative of suboptimal *ras* signaling. Quantitatively similar defects were observed in Th1 and Th2 cells. Since the ERK kinases regulate the *fos* component of the AP-1 transcription factor, these results suggest that soluble anti-CD3 IgG3 may induce less AP-1 than a cross-linked anti-CD3 stimulus (Cantrell, 1996).

Previous studies have shown that the anti-CD3 IgG3 mAb induced little PLCγ-1 phosphorylation, and calcium flux was not detectable by FACS analysis (Smith *et al.*, 1997). These results had suggested that the calcium signal delivered by anti-CD3 IgG3 must be very low. In a system using APLs, demonstration of a low amplitude calcium signal required sensitive video imaging techniques (Sloan-Lancaster *et al.*, 1996). The data presented here are the first indication that a partial TCR signal, characterized by an altered ratio of phospho-ζ and defective ZAP-70 phosphorylation (and a low level calcium signal), is sufficient to induce translocation of NF-ATc into the nucleus. NF-AT translocation occurred in both Th1 and Th2 cells stimulated by anti-CD3 IgG3. These results imply either that ZAP-70 phosphorylation is dispensable for this event, or that low levels are sufficient. In a recent study examining B cell signaling, Dolmetsch *et al.* (1997) showed that low levels of calcium resulted in NF-AT translocation (consistent with the inventors' findings), yet higher levels of calcium were required fro JNK and NF-KB activation (Dolmetsch *et al.*, 1997). Together with the MAP kinase data, these results suggest that stimulation by non-cross-linked anti-CD3 Abs may result in qualitatively and quantitatively different array of activated transcription factors than those induced by a cross-linked anti-CD3 stimulus.

A major question raised by the apparent similarity in anti-CD3 IgG3-mediated signal transduction in Th1 and Th2 cells is why the mAb selectively induced proliferation and unresponsiveness in specific subsets. The selective stimulation of proliferation by anti-CD3 IgG3 could reflect either quantitatively or qualitatively different requirements for driving IL-2 vs IL-4 transcription. For instance, it is possible that all the correct signals are being sent by anti-CD3 IgG3 at a reduced level, but the cytokine promoters have quantitatively different hierarchical thresholds for triggering. In the absence of cross-linking, anti-CD3 IgG3 induced 10-fold less IL-4 in Th2 clones. Anti-CD3 IgG3 stimulation of the Th1 clone, pGL10, resulted in two logs less IFN-γ production compared with immobilized anti-CD3 stimulation. The suboptimal levels of cytokine transcription factors induced by anti-CD3 IgG3 may fall below the threshold for effective IL-2 production. Alternatively, differential association of transcription factors with the IL-2 and IL-4 promoters may account for the disparate sensitivity (Tara *et al.*, 1995). This quantitative hypothesis is consistent with studies examining the effect of Ag dose on Th development. Several groups have reported

that extremely low levels of nominal Ag preferentially induced a Th2 subset phenotype, and that higher levels of Ag were required for Th1 differentiation (Constant *et al.*, 1995; Hosken *et al.*, 1995). However, low doses of Ag deliver proximal signals that are qualitatively different from the pattern of signaling triggered by APL (and thus anti-CD3 IgG3) (Sloan-Lancaster *et al.*, 1994; Madrenas *et al.*, 1997). Also, there is a complete lack of dose response to the anti-CD3 IgG3, wherein high amounts of mAb failed to induce proliferation in Th1 cells.

Alternatively, selective cytokine induction by anti-CD3 IgG3 could reflect qualitative differences in the transcription factors required for cytokine promoter activity. For instance, IL-4 transcription could be less dependent on triggering of all of the TCR-related signaling cascades. On a gross level, Th2 clones have been reported to produce IL-4 in response to calcium ionophores alone, whereas Th1 cells require another signal (*e.g.*, PMA) to produce IL-2 (Tamura *et al.*, 1993). Similarly, although anti-CD3 IgG3 induced Th2 proliferation, the mAb only elicited IL-2 production and proliferation in naive cells or Th1 clones in the presence of PMA. PMA may contribute by activating *ras* (thus enhancing AP-1 activity) or PKC (NF-KB). In fact, the NF-AT binding sites within the IL-2 promoter represent composite NF-AT/AP-1 sites, where AP-1 is required for activity (Rao, 1994). In contrast, it has been suggested that NF-AT, in the presence of other easily inducible factors (such as c-*maf*), may be sufficient to drive minimal IL-4 transcription. Unlike the IL-2 promoter, the IL-4 promoter contains NF-AT binding sites that do not require AP-1 (Rao, 1994). Despite the presence of these sites, it has been demonstrated that NF-AT and AP-1 greatly synergize in enhancing IL-4 transcription (Rooney *et al.*, 1994; Rooney *et al.*, 1995; Ho *et al.*, 1996). This difference between NF-AT activity in the presence or absence of AP-1 suggests a basis for the lower levels of IL-4 observed in the absence of anti-CD3 cross-linking. Taken together, these results suggest that the decreased level of MAP kinase activity (and thus AP-1) induced by non cross-linked anti-CD3 could be more deleterious for IL-2 than for IL-4 production. Furthermore, the NF-AT that translocates in response to non-cross-linked anti-CD3 may be sufficient for IL-4 production.

The data presented in this study have implications for how other TCR signaling-related therapies (such as APLs or nondepleting anti-CD4) may exert their protective effects *in vivo*, as well as for general mechanisms of tolerance induction. Consistent with the *in vivo* findings with FcR-nonbinding anti-CD3 mAbs, effective anti-CD4 therapy in transplantation and autoimmune diseases strongly correlates with Th deviation from a Th1 to a Th2 phenotype (Mouram *et al.*, 1995; Chu and Londci, 1996). It may be more than a coincidence that the proximal signals delivered by anti-CD3 IgG3 and under conditions of coreceptor blockade resemble each other (Hosken *et al.*, 1995). In a recent study, T cell lines and clones derived from mice injected with OK peptides displayed a Th0/Th2 phenotype, and adoptively prevented experimental allergic encephalomyelitis (Nicholson *et al.*, 1997). The results from the present study have shown that anti-CD3 IgG3 induces NF-AT translocation, but not efficient MAP kinase phosphorylation. Interestingly, in B cells, a toleragenic signal has been shown to consist of NF-AT and ERK activation, but no JNK kinase activation (Healy *et al.*, 1997). The selective activity of specific transcription factors (such as NF-AT) in the absence of others may translate into a toleragenic signal in multiple cell types. Thus, different models of altered Ag receptor signaling may reflect the use of common biochemical pathways that lead to tolerance as manifested by lymphocyte inactivation or cytokine deviation.

EXAMPLE 13
Human Treatment

A clinical protocol has been developed to facilitate the treatment of a patient using the immunomodulatory compositions described herein. In accordance with this protocol, patients having a need for the immunomodulatory intervention to effect a modulation of its immune response. Patients may, but need not have received previous immunotherapy. In a human patient in need of an immunomodulatory intervention of the present invention, the immunomodulatory compound is administered in an amount effective to modulate an immune system. Those of skill in the art will be able to employ methods of determining appropriate dosages know to those of skill and the teachings of this specification to determine appropriate dosage time-courses and amounts. It is anticipated the immunomodulatory compounds will be given in amounts ranging from 1 μg/kg to 20,000 μg/kg. Preferred ranges of compounds will be from 10 μg/kg to 2,000 μg/kg. More preferably, the compounds will be administered in a

range of from 10 μg/kg to 1,000 μg/kg, with 100 μg/kg to 400 μg/kg being considered particularly advantageous. The immunomodulatory compound may administered as a bolus or as a series of boluses. Such boluses may be delivered over a staggered time course with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 15, 17, 20, or more days between successive boluses. Upon election by the clinician, the regimen may be continued, six doses each two weeks, or on a less frequent (monthly, bimonthly, quarterly, *etc.*) basis.

* * *

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Arch Development Corporation
(B) STREET: 1101 East 58th Street
(C) CITY: Chicago
(D) STATE: IL
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 60637
(G) TELEPHONE: (512)418-3000
(H) TELEFAX: (512)474-7577

(ii) TITLE OF INVENTION: FC RECEPTOR NON-BINDING ANTI-CD3 MONOCLONAL ANTIBODIES DELIVER A PARTIAL TCR SIGNAL AND INDUCE CLONAL ANERGY

(iii) NUMBER OF SEQUENCES: 17

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: US Unknown
(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/044,084
(B) FILING DATE: 21-APR-1997

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2399 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:53..760

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1151..1186

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1308..1634

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1732..2052

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
ATCCTGGCAA AGATTGTAAT ACGACTCACT ATAGGGCGAA TTCGCCGCCA CC ATG          55
                                                          Met
                                                           1

GAA TGG AGC TGG GTC TTT CTC TTC TTC CTG TCA GTA ACT ACA GGT GTC       103
Glu Trp Ser Trp Val Phe Leu Phe Phe Leu Ser Val Thr Thr Gly Val
              5                  10                  15

CAC TCC CAG GTT CAG CTG GTG CAG TCT GGA GGA GGA GTC GTC CAG CCT       151
His Ser Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro
         20                  25                  30

GGA AGG TCC CTG AGA CTG TCT TGT AAG GCT TCT GGA TAC ACC TTC ACT       199
Gly Arg Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr
     35                  40                  45

AGA TAC ACA ATG CAC TGG GTC AGA CAG GCT CCT GGA AAG GGA CTC GAG       247
Arg Tyr Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu
 50                  55                  60                  65

TGG ATT GGA TAC ATT AAT CCT AGC AGA GGT TAT ACT AAC TAC AAT CAG       295
Trp Ile Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln
                 70                  75                  80

AAG GTG AAG GAC AGA TTC ACA ATT TCT AGA GAC AAT TCT AAG AAT ACA       343
Lys Val Lys Asp Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr
             85                  90                  95

GCC TTC CTG CAG ATG GAC TCA CTC AGA CCT GAG GAT ACC GGA GTC TAT       391
Ala Phe Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr
        100                 105                 110

TTT TGT GCT AGA TAT TAC GAT GAC CAC TAC TGT CTG GAC TAC TGG GGC       439
Phe Cys Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly
    115                 120                 125

CAA GGT ACC CCG GTC ACC GTG AGC TCA GCT TCC ACC AAG GGC CCA TCC       487
Gln Gly Thr Pro Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser
130                 135                 140                 145

GTC TTC CCC CTG GCG CCC TGC TCC AGG AGC ACC TCC GAG AGC ACA GCC       535
Val Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr Ala
                150                 155                 160

GCC CTG GGC TGC CTG GTC AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG       583
Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
            165                 170                 175

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC GTG CAC ACC TTC CCG GCT       631
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala
        180                 185                 190

GTC CTA CAG TCC TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG ACC GTG       679
Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
```

```
    195                 200                 205

CCC TCC AGC AGC TTG GGC ACG AAG ACC TAC ACC TGC AAC GTA GAT CAC      727
Pro Ser Ser Ser Leu Gly Thr Lys Thr Tyr Thr Cys Asn Val Asp His
210                 215                 220                 225

AAG CCC AGC AAC ACC AAG GTG GAC AAG AGA GTT GGTGAGAGGC CAGCACAGGG    780
Lys Pro Ser Asn Thr Lys Val Asp Lys Arg Val
                230                 235

AGGGAGGGTG TCTGCTGGAA GCCAGGCTCA GCCCTCCTGC CTGGACGCAC CCCGGCTGTG    840

CAGCCCCAGC CCAGGGCAGC AAGGCATGCC CCATCTGTCT CCTCACCCGG AGGCCTCTGA    900

CCACCCCACT CATGCTCAGG GAGAGGGTCT TCTGGATTTT TCCACCAGGC TCCCGGCACC    960

ACAGGCTGGA TGCCCCTACC CCAGGCCCTG CGCATACAGG GCAGGTGCTG CGCTCAGACC   1020

TGCCAAGAGC CATATCCGGG AGGACCCTGC CCCTGACCTA AGCCCACCCC AAAGGCCAAA   1080

CTCTCCACTC CCTCAGCTCA GACACCTTCT CTCCTCCCAG ATCTGAGTAA CTCCCAATCT   1140

TCTCTCTGCA GAG TCC AAA TAT GGT CCC CCA TGC CCA TCA TGC CCA            1186
          Glu Ser Lys Tyr Gly Pro Pro Cys Pro Ser Cys Pro
            1               5                  10

GGTAAGCCAA CCCAGGCCTC GCCCTCCAGC TCAAGGCGGG ACAGGTGCCC TAGAGTAGCC   1246

TGCATCCAGG GACAGGCCCC AGCCGGGTGC TGACGCATCC ACCTCCATCT CTTCCTCAGC   1306

A CCT GAG TTC CTG GGG GGA CCA TCA GTC TTC CTG TTC CCC CCA AAA        1352
  Pro Glu Phe Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys
    1               5                  10                  15

CCC AAG GAC ACT CTC ATG ATC TCC CGG ACC CCT GAG GTC ACG TGC GTG      1400
Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val
                20                  25                  30

GTG GTG GAC GTG AGC CAG GAA GAC CCC GAG GTC CAG TTC AAC TGG TAC      1448
Val Val Asp Val Ser Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr
            35                  40                  45

GTG GAT GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG      1496
Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu
        50                  55                  60

CAG TTC AAC AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC      1544
Gln Phe Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His
    65                  70                  75

CAG GAC TGG CTG AAC GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA      1592
Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys
80                  85                  90                  95

GGC CTC CCG TCC TCC ATC GAG AAA ACC ATC TCC AAA GCC AAA              1634
Gly Leu Pro Ser Ser Ile Glu Lys Thr Ile Ser Lys Ala Lys
                100                 105
```

```
GGTGGGACCC ACGGGGTGCG AGGGCCACAC GGACAGAGGC CAGCTCGGCC CACCCTCTGC    1694

CCTGGGAGTG ACCGCTGTGC CAACCTCTGT CCCTACA GGG CAG CCC CGA GAG CCA     1749
                                          Gly Gln Pro Arg Glu Pro
                                            1               5

CAG GTG TAC ACC CTG CCC CCA TCC CAG GAG GAG ATG ACC AAG AAC CAG      1797
Gln Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln
            10                  15                  20

GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAC CCC AGC GAC ATC GCC      1845
Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala
        25                  30                  35

GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG      1893
Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr
    40                  45                  50

CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AGG CTA      1941
Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu
55                  60                  65                  70

ACC GTG GAC AAG AGC AGG TGG CAG GAG GGG AAT GTC TTC TCA TGC TCC      1989
Thr Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser
                75                  80                  85

GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACA CAG AAG AGC CTC TCC      2037
Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser
            90                  95                  100

CTG TCT CTG GGT AAA TGAGTGCCAG GGCCGGCAAG CCCCCGCTCC CCGGGCTCTC      2092
Leu Ser Leu Gly Lys
        105

GGGGTCGCGC GAGGATGCTT GGCACGTACC CCGTCTACAT ACTTCCCAGG CACCCAGCAT    2152

GGAAATAAAG CACCCACCAC TGCCCTGGGC CCCTGTGAGA CTGTGATGGT TCTTTCCACG    2212

GGTCAGGCCG AGTCTGAGGC CTGAGTGACA TGAGGGAGGC AGAGCGGGTC CCACTGTCCC    2272

CACACTGGCC CAGGCGTTGC AGTGTGTCCT GGGCCACCTA GGGTGGGGCT CAGCCAGGGG    2332

CTCCCTCGGC AGGGTGGGGC ATTTGCCAGC GTGGCCCTCC CTCCAGCAGC AGGACTCTAG    2392

AGGATCC                                                              2399
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 236 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Glu Trp Ser Trp Val Phe Leu Phe Phe Leu Ser Val Thr Thr Gly
  1               5                  10                  15

Val His Ser Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln
             20                  25                  30

Pro Gly Arg Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe
         35                  40                  45

Thr Arg Tyr Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
     50                  55                  60

Glu Trp Ile Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn
 65                  70                  75                  80

Gln Lys Val Lys Asp Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn
                 85                  90                  95

Thr Ala Phe Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val
            100                 105                 110

Tyr Phe Cys Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp
        115                 120                 125

Gly Gln Gly Thr Pro Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
    130                 135                 140

Ser Val Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr
145                 150                 155                 160

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
                165                 170                 175

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
            180                 185                 190

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
        195                 200                 205

Val Pro Ser Ser Ser Leu Gly Thr Lys Thr Tyr Thr Cys Asn Val Asp
    210                 215                 220

His Lys Pro Ser Asn Thr Lys Val Asp Lys Arg Val
225                 230                 235
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Glu Ser Lys Tyr Gly Pro Pro Cys Pro Ser Cys Pro
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Pro Glu Phe Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro
 1               5                  10                  15

Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
            20                  25                  30

Val Asp Val Ser Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val
        35                  40                  45

Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln
    50                  55                  60

Phe Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln
65                  70                  75                  80

Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly
                85                  90                  95

Leu Pro Ser Ser Ile Glu Lys Thr Ile Ser Lys Ala Lys
            100                 105
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Gln Glu
 1               5                  10                  15

Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
            20                  25                  30

Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu
        35                  40                  45

Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe
    50                  55                  60
```

```
Phe Leu Tyr Ser Arg Leu Thr Val Asp Lys Ser Arg Trp Gln Glu Gly
 65                  70                  75                  80

Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr
                 85                  90                  95

Thr Gln Lys Ser Leu Ser Leu Ser Leu Gly Lys
            100                 105
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Gln Ile Val Leu Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly
1               5                   10                  15

Glu Lys Val Thr Met Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met
            20                  25                  30

Asn Trp Tyr Gln Gln Lys Ser Gly Thr Ser Pro Lys Arg Trp Ile Tyr
        35                  40                  45

Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ala His Phe Arg Gly Ser
    50                  55                  60

Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Gly Met Glu Ala Glu
65                  70                  75                  80

Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser Asn Pro Phe Thr
                85                  90                  95

Phe Gly Ser Gly Thr Lys Leu Glu Ile Asn Arg
            100                 105
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 96 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Gln Ala Ser Gln Asp Ile Ile Lys Tyr
```

```
                20                  25                  30

Leu Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr Glu Ala Ser Asn Leu Gln Ala Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Gln Ser Leu Pro Tyr
                85                  90                  95
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met
            20                  25                  30

Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr
        35                  40                  45

Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
    50                  55                  60

Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro Glu
65                  70                  75                  80

Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser Asn Pro Phe Thr
                85                  90                  95

Phe Gly Gln Gly Thr Lys Leu Gln Ile Thr Arg
            100                 105
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                  10                  15

Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met
            20                  25                  30

Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Lys Arg Trp Ile Tyr
        35                  40                  45

Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
    50                  55                  60

Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro Glu
65                  70                  75                  80

Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser Asn Pro Phe Thr
                85                  90                  95

Phe Gly Gln Gly Thr Lys Leu Gln Ile Thr Arg
            100                 105
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Ala Arg Pro Gly Ala
1               5                  10                  15

Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30

Thr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
        35                  40                  45

Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Asp Lys Ala Thr Leu Thr Thr Asp Lys Ser Ser Ser Thr Ala Tyr
65                  70                  75                  80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Thr Leu Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 126 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                  10                  15

Ser Leu Arg Leu Ser Cys Ser Ser Ser Gly Phe Ile Phe Ser Ser Tyr
            20                  25                  30

Ala Met Tyr Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ala Ile Ile Trp Asp Asp Gly Ser Asp Gln His Tyr Ala Asp Ser Val
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Phe
65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
                85                  90                  95

Ala Arg Asp Gly Gly His Gly Phe Cys Ser Ser Ala Ser Cys Phe Gly
            100                 105                 110

Pro Asp Tyr Trp Gly Gln Gly Thr Pro Val Thr Val Ser Ser
        115                 120                 125
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                  10                  15

Ser Leu Arg Leu Ser Cys Ser Ser Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30

Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ala Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Phe
    50                  55                  60
```

```
Lys Asp Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Phe
65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
                85                  90                  95

Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Pro Val Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
            20                  25                  30

Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
        35                  40                  45

Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Ser Thr Ala Phe
65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Pro Val Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1 5 10 15

Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
20 25 30

Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
35 40 45

Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Phe
50 55 60

Lys Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Ser Thr Ala Phe
65 70 75 80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
100 105 110

Thr Pro Val Thr Val Ser Ser
115

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TCCAGATGTT AACTGCTCAC 20

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CAGGGGCCAG TGGATGGATA GAC 23

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCCGCCACC 9

CLAIMS:

1. A method of modulating the immune system of a mammal which comprises:

obtaining an immunomodulatory compound that selectively induces ξ chain tyrosine phosphorylation of a p21 form of ξ of the TCR complex without induction of the highly phosphorylated p23 form of ξ and triggers ZAP-70 association, but does not induce tryrosine phosphorylation of associated ZAP-70 tyrosine kinase;

combining the immunomodulatory compound in a pharmaceutically acceptable vehicle; and

administering the resulting composition to the mammal in amounts effective to modulate an immune system.

2. The method of claim 1, wherein the immunomodulatory compound selectively inactivates Th1 and/or IL2 producing T cells while promoting Th2 type T cells.

3. The method of claim 1, wherein the immunomodulatory compound is a small molecule.

4. The method of claim 1, wherein the immunomodulatory compound is a monoclonal antibody.

5. The method of claim 4, wherein the monoclonal antibody is a Fc receptor non-binding anti-CD3 monoclonal antibody.

6. The method of claim 5, wherein the Fc receptor non-binding anti-CD3 monoclonal antibody comprises a complementary determining region of murine OKT3, a human IgG variable framework, and a human IgG constant region, the constant region comprising a point-mutation to alanine at position 234.

7. The method of claim 5, wherein the Fc receptor non-binding anti-CD3 monoclonal antibody comprises a complementary determining region of murine OKT3, a human IgG variable framework, and a human IgG constant region, the constant region comprising a point-mutation to alanine at position 235.

8. The method of claim 5, wherein the Fc receptor non-binding anti-CD3 monoclonal antibody comprises a complementary determining region of murine OKT3, a human IgG variable framework, and a human IgG constant region, the constant region comprising a double point-mutation to alanine at position 234 and alanine at position 235.

9. The method of claim 8, wherein the variable framework and constant region of the Fc receptor non-binding anti-CD3 monoclonal antibody are of either a human IgG4 or a human IgG1.

10. The method of claim 5, wherein the Fc receptor non-binding anti-CD3 monoclonal antibody comprises the variable framework and constant region are of a human IgG4 and a mutation from a phenylalanine to an alanine at position 234.

11. The method of claim 10, wherein the Fc receptor non-binding anti-CD3 monoclonal antibody comprises the variable framework and constant region are of a human IgG4 and a mutation from a leucine to an alanine at position 235

12. The method of claim 10, wherein the Fc receptor non-binding anti-CD3 monoclonal antibody comprises the variable framework and constant region are of a human IgG1 and a mutation from a leucine to an alanine at position 234.

13. The method of claim 10, wherein the Fc receptor non-binding anti-CD3 monoclonal antibody comprises the variable framework and constant region are of a human IgG1 and a mutation from a leucine to an alanine at position 235.

14. The method of claim 4, wherein the monoclonal antibody is directed against non-polymorphic TcR-associated CD3 chains, g, d, e or l.

15. The method of claim 1, wherein the mammal is receiving a hematopoietic tissue transplant.

16. The method of claim 15, wherein said mammal is a human.

17. The method of claim 1, wherein the immunomodulatory compound is administered in an amount from 10 mg/kg to 2,000 mg/kg.

18. The method of claim 1, wherein the immunomodulatory compound is administered in an amount from 10 mg/kg to 1,000 mg/kg.

19. The method of claim 1, wherein the immunomodulatory compound is delivered in an amount from 100 mg/kg to 400 mg/kg.

20. The method of claim 1, wherein the immunomodulatory compound is administered as a bolus.

21. The method of claim 1, wherein the immunomodulatory compound is administered as a series of boluses.

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LIGHT CHAIN

```
                10        20        30        40        50
Okt3v1  QIVLTQSPAIMSASPGEKVTMTCSASS-SVSYMNWYQQKSGTSPKRWIYDTSKLAS  SEQ ID NO:6
REI     DIQMTQSPSSLSASVGDRVTITCQASQDIIKYLNWYQQTPGKAPKLLIYEASNLQA  SEQ ID NO:7
gL      ....................SA.S-SVS.M.............DT.K.AS  SEQ ID NO:8
gLC     ....................SA.S-SVS.M.........RW..DT.K.AS  SEQ ID NO:9

          60        70        80        90        100   108
OKT3VL  GVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEINR      SEQ ID NO:6
REI     GVPSRFSGSGSGTDYTFTISSLQPEDIATYYCQQYQSLPYTFGQGTKLQITR      SEQ ID NO:7
gL      ..................................WS.N.F.........      SEQ ID NO:8
gLC     ..........................................      SEQ ID NO:9
```

FIG. 1A

HEAVY CHAIN

```
                  10          20          30
Okt3vh   QVQLQQSGAELARPGASVKMSCKASGYTFTRY      SEQ ID NO:10
KOL      QVQLVESGGGVVQPGRSLRLSCSSSGFIFSSY      SEQ ID NO:11
gH       ..............................YT.TR.   SEQ ID NO:12
gHA      .....Q..............KA..YT.TR.        SEQ ID NO:13
gHG      .....Q..............KA..YT.TR.        SEQ ID NO:14

                  40          50          60
Okt3vh   TMHWVKQRPGQGLEWIGYINPSRGYTNYNQKF      SEQ ID NO:10
KOL      AMYWVRQAPGKGLEWVAIIWDDGSDQHYADSV      SEQ ID NO:11
gH       T.H..............Y.NPSRGYTN.NQKF      SEQ ID NO:12
gHA      T.H............IGY.NPSRGYTN.NQK.       SEQ ID NO:13
gHG      T.H............IGY.NPSRGTYN.NQK.       SEQ ID NO:14

                  70          80          90
Okt3vh   KDKATLTTDKSSSTAYMQLSSLTSEDSAVYYC      SEQ ID NO:10
KOL      KGRFTISRDNSKNTLFLQMDSLRPEDTGVYFC      SEQ ID NO:11
gH       .D..............................      SEQ ID NO:12
gHA      .D.....T.K..S.A............A..Y.       SEQ ID NO:13
gHG      .D............A...............        SEQ ID NO:14

                 100         110         120  126
Okt3vh   ARYYDDHYCL-------DYWGQGTTLTVSS        SEQ ID NO:10
KOL      ARDGGHGFCSSASCFGPDYWGQGTPVTVSS        SEQ ID NO:11
gH       ..YYDDHY.L-------..............       SEQ ID NO:12
gHA      ..YYDDHY.L-------..............       SEQ ID NO:13
gHG      ..YYDDHY.L-------..............       SEQ ID NO:14
```

FIG. 1B

```
     5   10   15   20   25   30   35   40   45   50   55
         *    *    *    *    *    *    *    *    *
ATCCTGGCAA AGATTGTAAT ACGACTCACT ATAGGGCGAA TTCGCCGCCA CC ATG GAA
                                                    Met Glu>
                                                     ___a___>

    60   65   70   75   80   85   90   95  100  105
     *    *    *    *    *    *    *    *    *
TGG AGC TGG GTC TTT CTC TTC TTC CTG TCA GTA ACT ACA GGT GTC CAC
Trp Ser Trp Val Phe Leu Phe Leu Ser Val Thr Thr Gly Val His>
___a___a___TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_a___a___a___>

   110  115  120  125  130  135  140  145  150
     *    *    *    *    *    *    *    *
TCC CAG GTT CAG CTG GTG CAG TCT GGA GGA GGA GTC GTC CAG CCT GGA
Ser Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly>
___a___a___TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_a___a___a___>

155 160  165  170  175  180  185  190  195  200
     *    *    *    *    *    *    *    *
AGG TCC CTG AGA CTG TCT TGT AAG GCT TCT GGA TAC ACC TTC ACT AGA
Arg Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg>
___a___a___TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_a___a___a___>
```

FIG. 2A

```
205     210     215     220     225     230     235     240     245     250
          *               *               *               *               *
TAC ACA ATG CAC TGG GTC AGA CAG GCT CCT GGA AAG GGA CTC GAG TGG
Tyr Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Aly Leu Glu Trp>
___a___a___TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_a___a___a___>

   255     260     265     270     275     280     285     290     295
             *               *               *               *
ATT GGA TAC ATT AAT CCT AGC AGA GGT TAT ACT AAC TAC AAT CAG AAG
Ile Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys>
___a___a___TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_a___a___a___>

300     305     310     315     320     325     330     335     340  345
 *               *               *               *               *
GTG AAG GAC AGA TTC ACA ATT TCT AGA GAC AAT TCT AAG AAT ACA GCC
Val Lys Asp Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Ala>
___a___a___TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_a___a___a___>

  350   355     360     365   370     375     380   385     390
    *               *               *               *               *
TTC CTG CAG ATG GAC TCA CTC AGA CCT GAG GAT ACC GGA GTC TAT TTT
Phe Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe>
___a___a___TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_a___a___a___>
```

FIG. 2B

```
395 400    405    410   415    420    425   430    435    440
     *           *          *          *          *
TGT GCT AGA TAT TAC GAT GAC CAC TAC TGT CTG GAC TAC TGG GGC CAA
Cys Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln>
___a___a___TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_a___a___a___>

     >SEQED_(include)_of:_ja91.ins_check:_5694_from:_1_to:_2153
                                        |
445    450    445   460    465    |470   475    480    485   490
        *          *              |  *          *          *
GGT ACC CCG GTC ACC GTG AGC TCA GCT | TCC ACC AAG GGC CCA TCC GTC
Gly Thr Pro Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val>
___a___a___TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_a___a___a___>

  495    500   505    510    515   520    525    530   535
          *          *          *          *
TTC CCC CTG GCG CCC TGC TCC AGG AGC ACC TCC GAG AGC ACA GCC GCC
Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr Ala Ala>
___a___a___TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_a___a___a___>
```

FIG. 2C

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```
540   545   550   555   560   565   570   575   580   585
 *           *           *           *           *

CTG GGC TGC CTG GTC AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG TCG
Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser>
__a__a__TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_a__a__a__>

  590   595   600   605   610   615   620   625   630
   *           *           *           *           *

TGG AAC TCA GGC GCC CTG ACC AGC GGC GTG CAC ACC TTC CCG GCT GTC
Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val>
__a__a__TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_a__a__a__>

635 640   645   650   655   660   665   670   675   680
     *           *           *           *           *

CTA CAG TCC TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG ACC GTG CCC
Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro>
__a__a__TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_a__a__a__>

685   690   695   700   705   710   715   720   725   730
       *           *           *           *           *

TCC AGC AGC TTG GGC ACT AAG ACC TAC ACC TGC AAC GTA GAT CAC AAG
Ser Ser Ser Leu Gly Thr Lys Thr Tyr Thr Cys Asn Val Asp His Lys>
__a__a__TRANSLATION OF OKT3 hc igg4 12/4/92 [a]_a__a__a__>
```

FIG. 2D

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```
  735   740  745   750   755   760  765 770   775 780
          *          *          *        *        *
CCC AGC AAC ACC AAG GTG GAC AAG AGA GTT GGTGAGAGGC CAGCACAGGG
pro Ser Asn Thr Lys Val Asp Lys Arg Val>
__TRANSLATION OF OKT3 HC IGG4 12/4___>

  785 790   795 800   805 810   815 820   825 830   835 840
        *         *         *         *         *         *
AGGGAGGGTG TCTGCTGGAA GCCAGGCTCA GCCCTCCTGC CTGGACGCAC CCCGGCTGTG
  845 850   855 860   865 870   875 880   885 890   895 900
        *         *         *         *         *         *
CAGCCCCAGC CCAGGGCAGC AAGGCATGCC CCATCTGTCT CCTCACCCGG AGGCCTCTGA

  905 910   915 920   925 930   935 940   945 950   955 960
        *         *         *         *         *         *
CCACCCCACT CATGCTCAGG GAGAGGGTCT TCTGGATTTT TCCACCAGGC TCCCGGCACC

  965 970   975 980   985 990   995 1000  1005 1010 1015 1020
        *         *         *         *         *         *
ACAGGCTGGA TGCCCCTACC CCAGGCCCTG CGCATACAGG GCAGGTGCTG CGCTCAGACC
```

FIG. 2E

```
1025 1030  1035 1040  1045 1050  1055 1060  1065 1070  1075 1080
          *          *          *          *          *          *
TGCCAAGAGC CATATCCGGG AGGACCCTGC CCCTGACCTA AGCCCACCCC AAAGGCCAAA
 1085 1090  1095 1100  1105 1110  1115 1120  1125 1130  1135 1140
          *          *          *          *          *          *
CTCTCCACTC CCTCAGCTCA GACACCTTCT CTCCTCCCAG ATCTGAGTAA CTCCCAATCT
 1145 1150   1155   1160  1165   1170   1175  1180   1185   1190
          *            *           *           *           *
TCTCTCTGCA GAG TCC AAA TAT GGT CCC CCA TGC CCA TCA TGC CCA GGTA
           Glu Ser Lys Tyr Gly Pro Pro Cys Pro Ser Cys Pro>
           ___TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_b___>
1195  1200  1205 1210  1215 1220  1225 1230  1235 1240  1245 1250
          *          *          *          *          *          *
AGCCAACCCA GGCCTCGCCC TCCAGCTCAA GGCGGGACAG GTGCCCTAGA GTAGCCTGCA
 1255 1260  1265 1270  1275 1280  1285 1290  1295 1300  1305  1310
          *          *          *          *          *          *
TCCAGGGACA GGCCCCAGCC GGGTGCTGAC GCATCCACCT CCATCTCTTC CTCAGCA CCT
                                                              Pro>
                                                              ___>
```

FIG. 2F

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```
  1315   1320  1325   1330   1335  1340   1345   1350  1355
           *           *           *           *

GAC TCC CTG GGG GGA CCA TCA GTC TTC CTG TTC CCC CCA AAA CCC AAG
Glu Phe Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys>
__c__c__TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_c__c__c__>

1360 1365  1370   1375   1380  1385   1390   1395  1400   1405
 *           *           *           *           *

GAC ACT CTC ATG ATC TCC CGG ACC CCT GAG GTC ACG TGC GTG GTG GTG
Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val>
__c__c__TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_c__c__c__>

 1410  1415   1420   1425  1430   1435   1440  1445   1450
    *           *           *           *           *

GAC GTG AGC CAG GAA GAC CCC GAG GTC CAG TTC AAC TGG TAC GTG GAT
Asp Val Ser Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp>
__c__c__TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_c__c__c__>

1455 1460 1465   1470  1475   1480   1485  1490   1495   1500
      *           *           *           *           *

GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TTC
Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe>
__c__c__TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_c__c__c__>
```

FIG. 2G

1505 1550 1515 1520 1525 1530 1535 1540 1545 1550

* * * * *

AAC AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC

Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp>

__c__c__TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_c__c__c__>

1555 1560 1565 1570 1575 1580 1585 1590 1595

* * * *

TGG CTG AAC GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GGC CTC

Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu>

__c__c__TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_c__c__c__>

1600 1605 1610 1615 1620 1625 1630 1635 1640 1645 1650

* * * * * *

CCG TCC TCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGTGGG ACCCACGGGG

Pro Ser Ser Ile Glu Lys Thr Ile Ser Lys Ala Lys>

__TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_c__>

1655 1660 1665 1670 1675 1680 1685 1690 1695 1700 1705 1710

* * * * * *

TGCGAGGGCC ACACGGACAG AGGCCAGCTC GGCCCACCCT CTGCCCTGGG AGTGACCGCT

FIG. 2H

```
 1715 1720 1725  1730    1735  1740   1745   1750  1755   1760
        *         *          *           *           *
GTGCCAACCT CTGTCCCTAC A GGG CAG CCC CGA GAG CCA CAG GTG TAC ACC
                        Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr>
                        ___TRANSLATION OF OKT3 HC IGG4 12/4___>

1765  1770   1775   1780  1785   1790   1795  1800c  1805
         *           *          *           *
CTG CCC CCA TCC CAG GAG GAG ATG ACC AAG AAC CAG GTC AGC CTG ACC
Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr>
___d___d___TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_d___d___d___>

1810 1815 1820   1825  1830   1835   1840  1845   1850   1855
*           *          *           *           *
TGC CTG GTC AAA GGC TTC TAC CCC AGC GAC ATC GCC GTG GAG TGG GAG
Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu>
___d___d___TRANSACTION OF OKT3 HC IGG4 12/4/92 [A]_d___d___d___>

1860  1865   1870  1875   1880   1885  1890   1895   1900  1905
  *           *          *           *           *
AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG
Ser Asn Gly Gln Pro Glu Asn Asn TyrLys Thr Thr Pro Pro Val Leu>
___d___d___TRANSACTION OF OKT3 HC IGG4 12/4/92 [A]_d__d___d___>
```

FIG. 2I

```
  1910   1915  1920   1925   1930  1935  1940    1945  1950
    *            *             *           *           *
GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AGG CTA ACC GTG GAC AAG
Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr Val Asp Lys>
___d___d___TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_d___d___d___>

1955 1960  1965   1970   1975  1980   1985   1990  1995   2000
       *           *            *            *           *
AGC AGG TGG CAG GAG GGG AAT GTC TTC TCA TGC TCC GTG ATG CAT GAG
Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val Met His Glu>
___d___d___TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_d___d___d___>

 2005  2010   2015   2020  2025   2030   2035  2040   2045
         *            *           *           *
GCT CTG CAC AAC CAC TAC ACA CAG AAG AGC CTC TCC CTG TCT CTG GGT
Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Leu Gly>
___d___d___TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_d___d___d___>

2050 2055 2060 2065 2070  2075 2080 2085  2090  2095 2100
*           *           *           *           *           *
AAA TGA GTGCC AGGGCCGGCA AGCCCCCGCT CCCCGGGCTC TCGGGGTCGC
Lys ***>
___d___>
```

FIG. 2J

```
2105 2110 2115 2120 2125 2130 2135 2140 2145 2150 2155 2160
     *    *    *    *    *    *    *    *    *    *
GCGAGGATGC TTGGCACGTA CCCCGTCTAC ATACTTCCCA GGCACCCAGC ATGGAAATAA

2165 2170 2175 2180 2185 2190 2195 2200 2205 2210 2215 2220
     *    *    *    *    *    *    *    *    *    *
AGCACCCACC ACTGCCCTGG GCCCCTGTGA GACTGTGATG GTTCTTTCCA CGGGTCAGGC

2225 2230 2235 2240 2245 2250 2255 2260 2265 2270 2275 2280
     *    *    *    *    *    *    *    *    *    *
CGAGTCTGAG GCCTGAGTGA CATGAGGGAG GCAGAGCGGG TCCCACTGTC CCCACACTGG

2285 2290 2295 2300 2305 2310 2315 2320 2325 2330 2335 2340
     *    *    *    *    *    *    *    *    *    *
CCCAGGCGTT GCAGTGTGTC CTGGGCCACC TAGGGTGGGG CTCAGCCAGG GGCTCCCTCG

2345 2350 2355 2360 2365 2370 2375 2380 2385 2390 2395
     *    *    *    *    *    *    *    *    *
GCAGGGTGGG GCATTTGCCA GCGTGGCCCT CCCTCCAGCA GCAGGACTCT AGAGGATCC
```

FIG. 2K

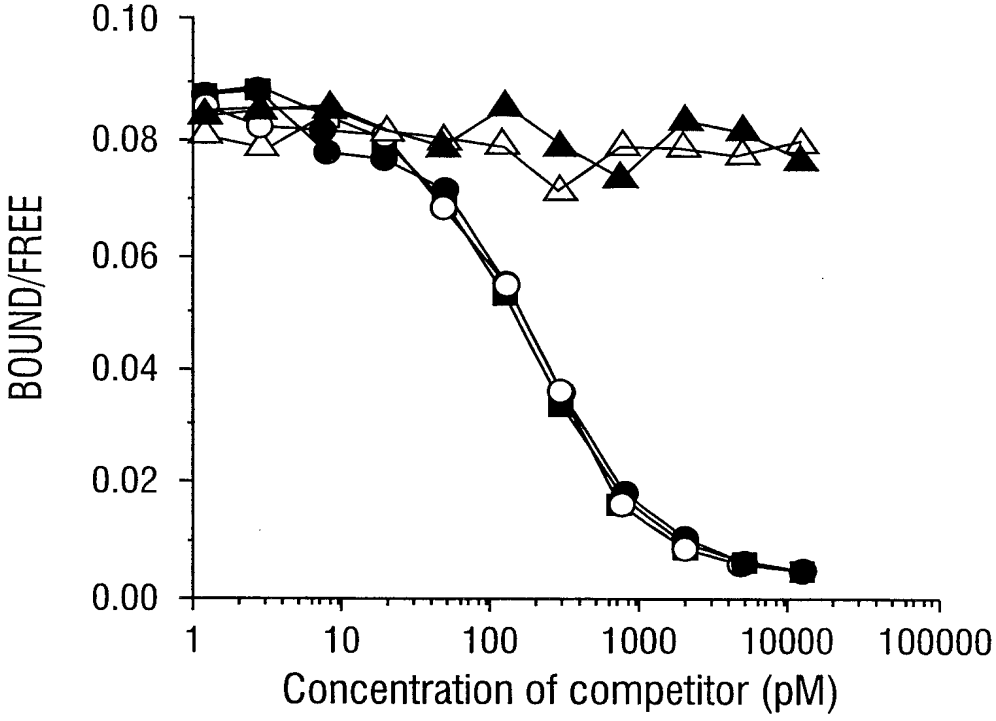
0.10
0.08
0.06
0.04
0.02
0.00
BOUND/FREE
1
10
100
1000
10000
100000
Concentration of competitor (pM)

FIG. 3A

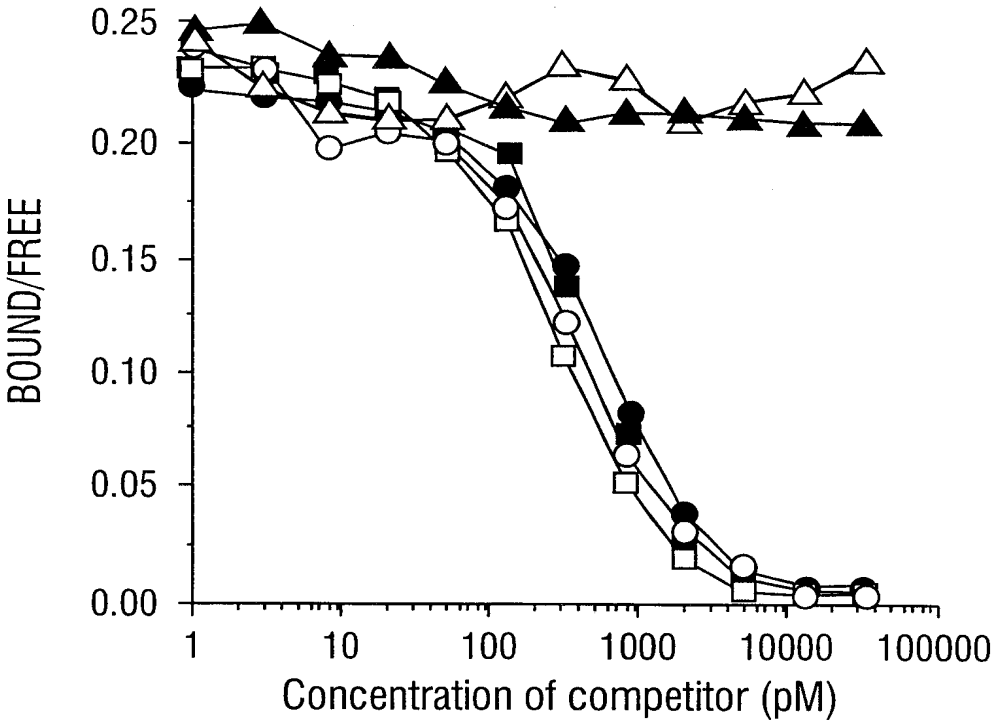
0.25
0.20
0.15
0.10
0.05
0.00
BOUND/FREE
1
10
100
1000
10000
100000
Concentration of competitor (pM)

FIG. 3B

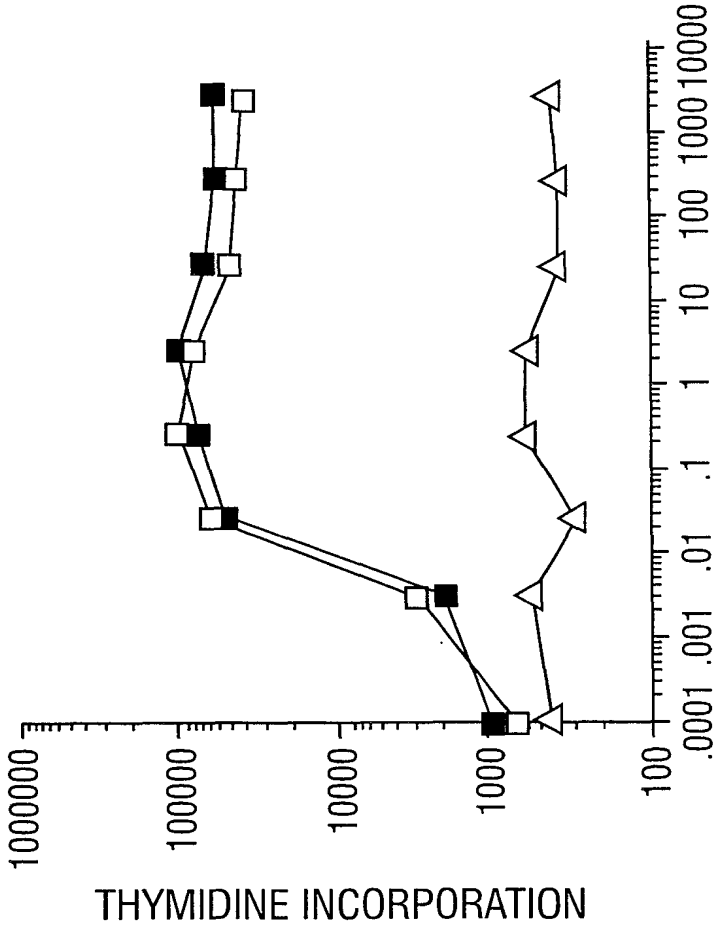
THYMIDINE INCORPORATION
1000000
100000
10000
1000
100
ANTIBODY (ng/mL)
.0001 .001 .01 .1 1 10 100 1000 10000

FIG. 4

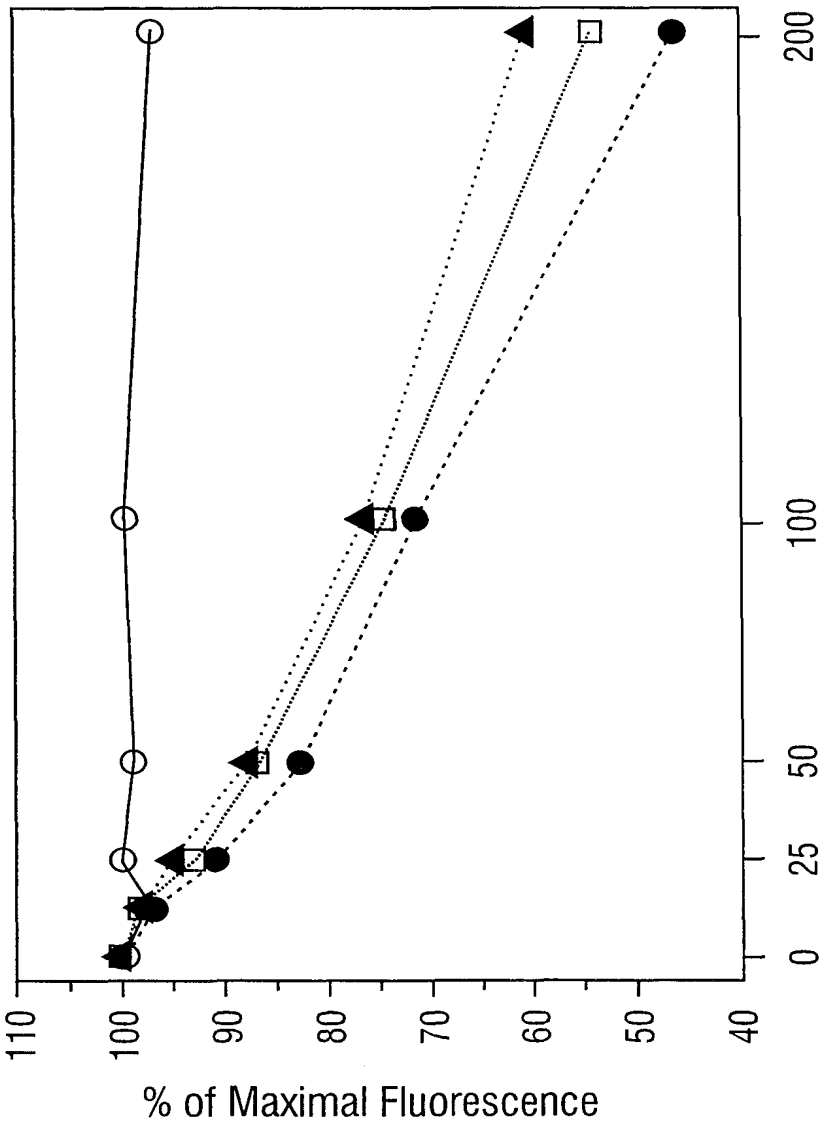
110
100
90
80
70
60
50
40
% of Maximal Fluorescence
0
25
50
100
200
[mAb] (ng/tube)

FIG. 5

N-terminal of CH2 domain

Isotype	234	235	236	237	238	239
h1gG1 h1gG3	Leu	Leu	Gly	Gly	Pro	Ser
m1gG2a						
h1G4	Phe	Leu	Gly	Gly	Pro	Ser
h1gG2	Val	---	Ala	Gly	Pro	Ser
m1gG2b	Leu	Glu	Gly	Gly	Pro	Ser
h1gG1A/A	Ala	Ala	Gly	Gly	Pro	Ser
h1gG4A/A	Ala	Ala	Gly	Gly	Pro	Ser

FIG: 6

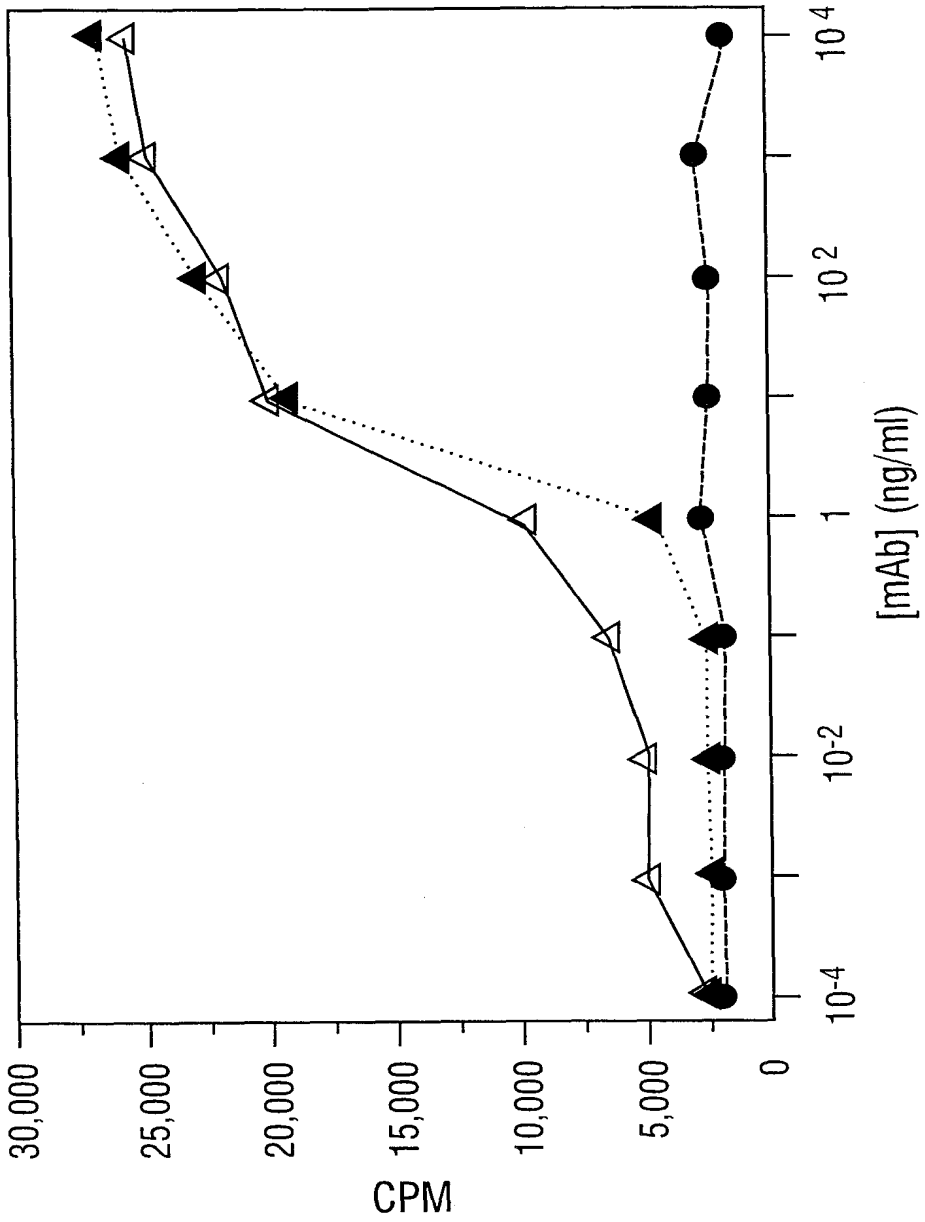
30,000
25,000
20,000
15,000
10,000
5,000
0
CPM
10-4
10-2
1
10 2
10 4
[mAb] (ng/ml)

FIG. 7

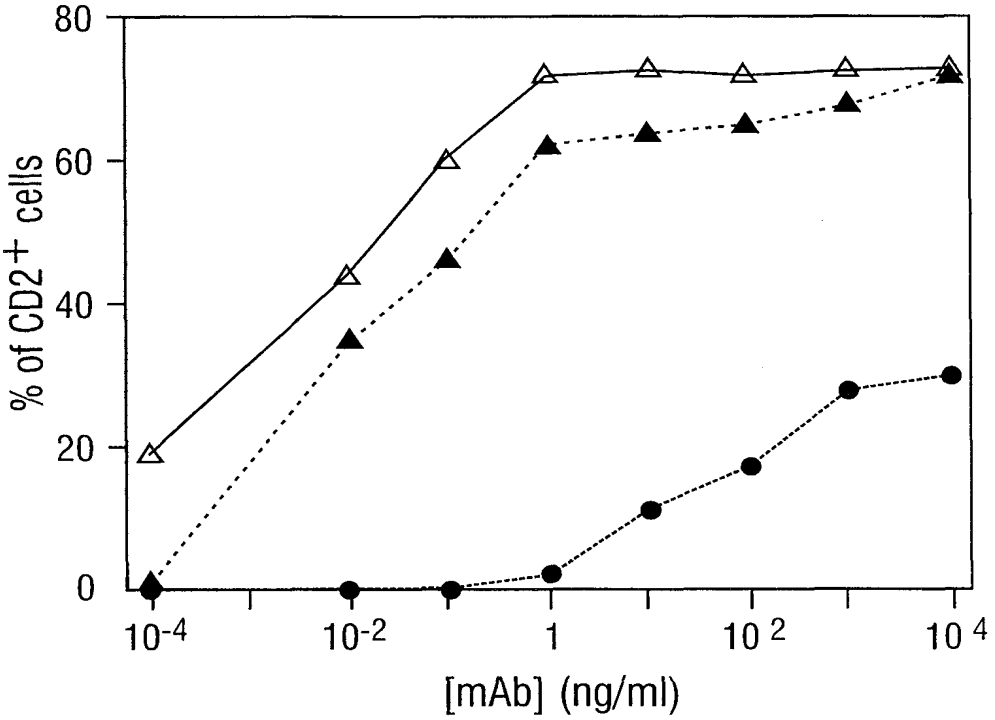
80
60
40
20
0
% of CD2+ cells
10-4
10-2
1
10 2
10 4
[mAb] (ng/ml)

FIG. 8A

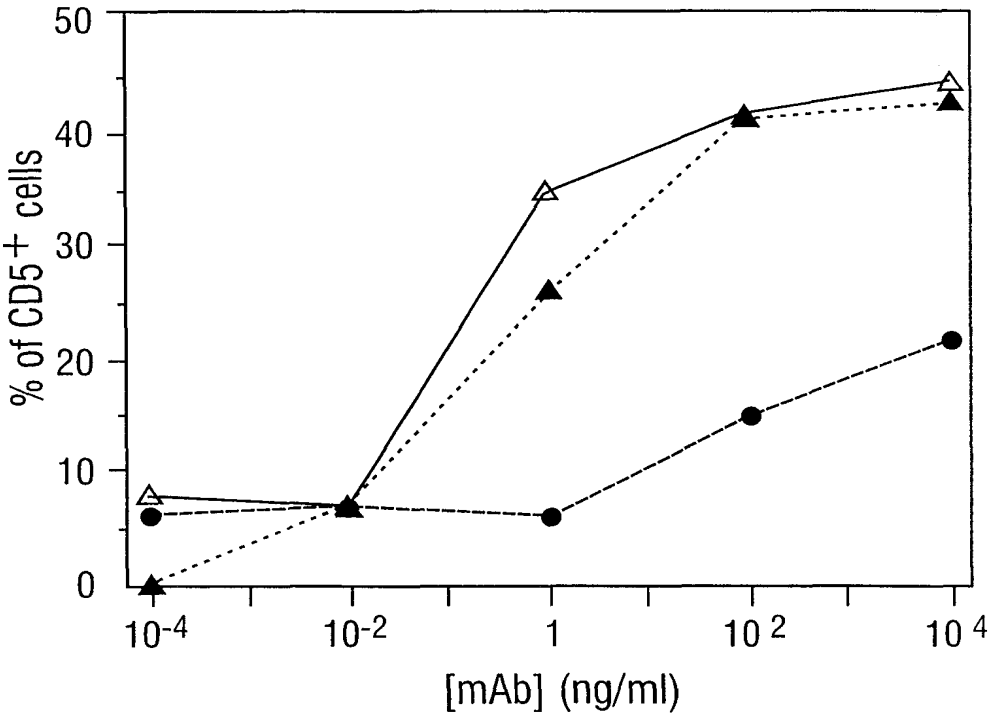
50
40
30
20
10
0
% of CD5+ cells
10-4
10-2
1
10 2
10 4
[mAb] (ng/ml)

FIG. 8B

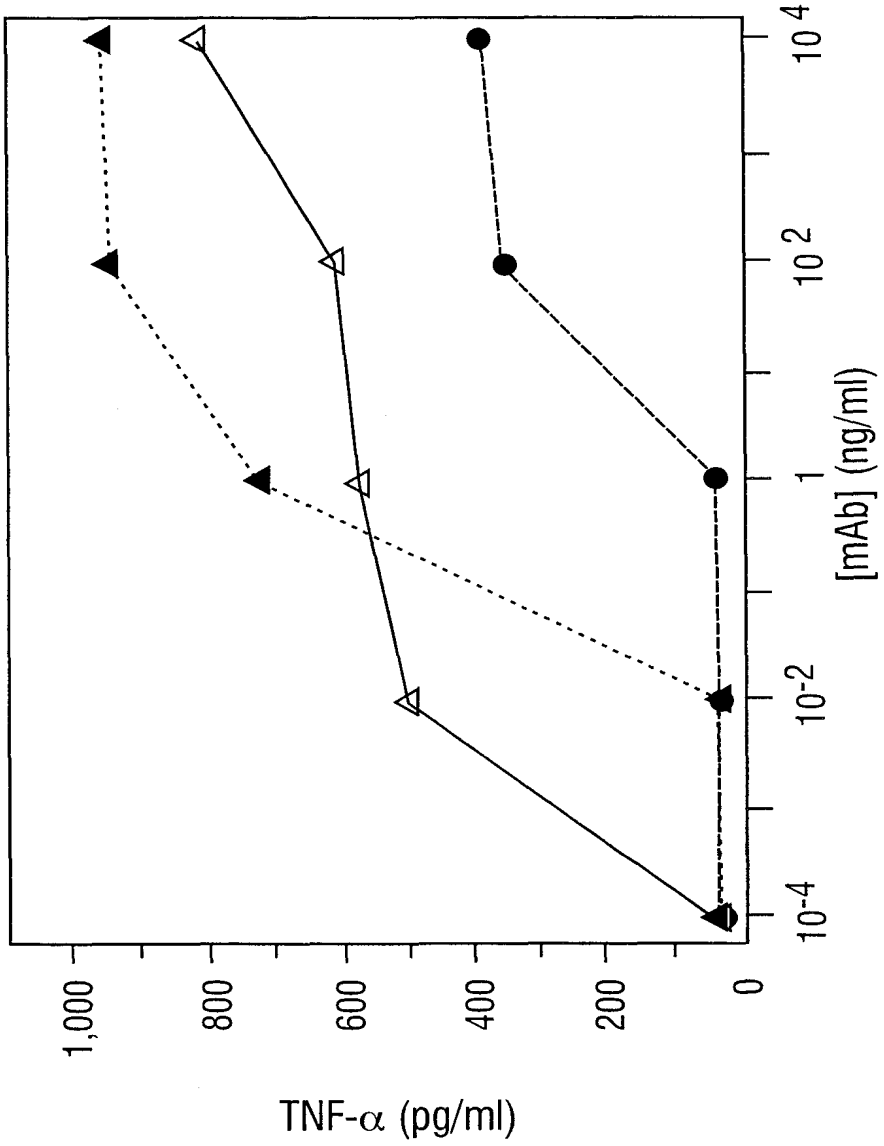

FIG. 9

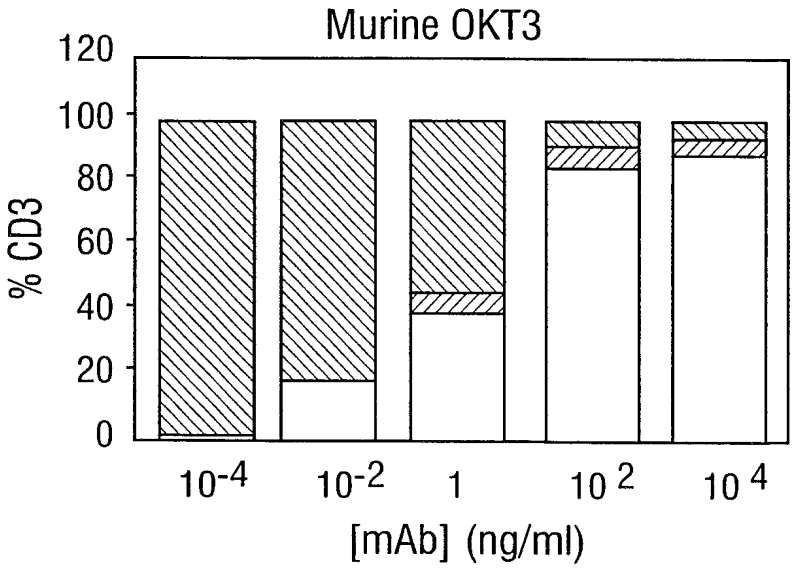

FIG. 10A

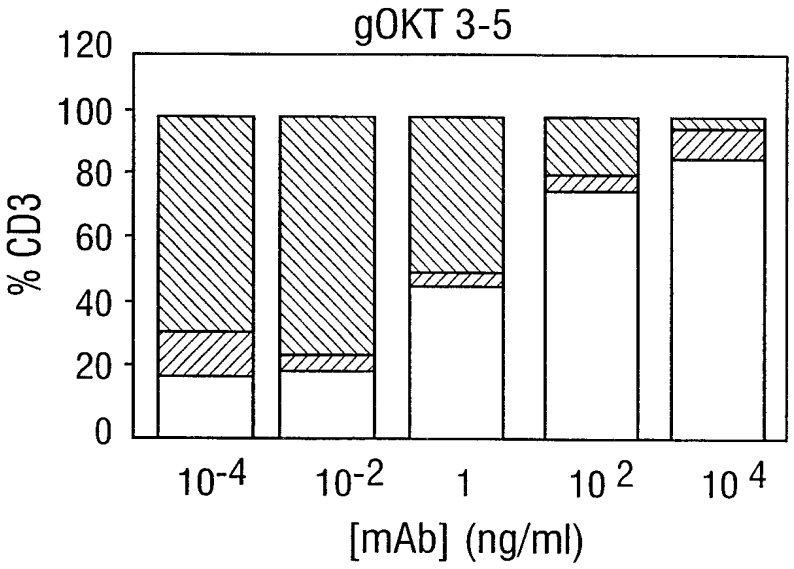

FIG. 10B

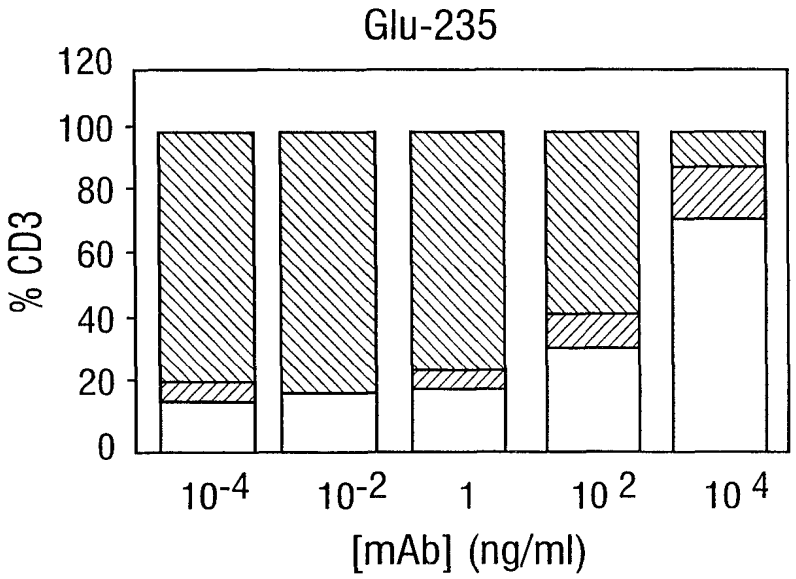

FIG. 10C

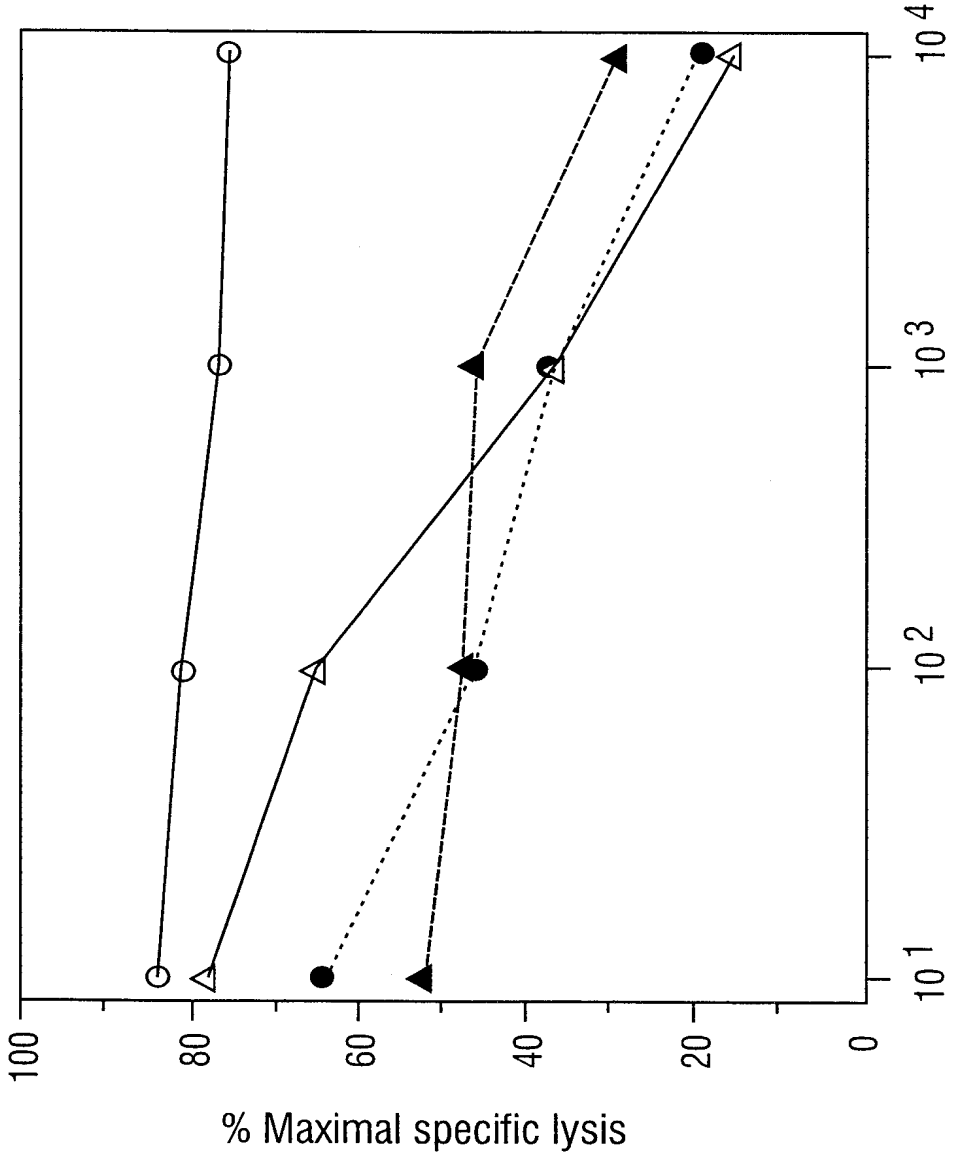
100
80
60
40
20
0
% Maximal specific lysis
10 1
10 2
10 3
10 4
[mAb] (ng/ml)

FIG. 11

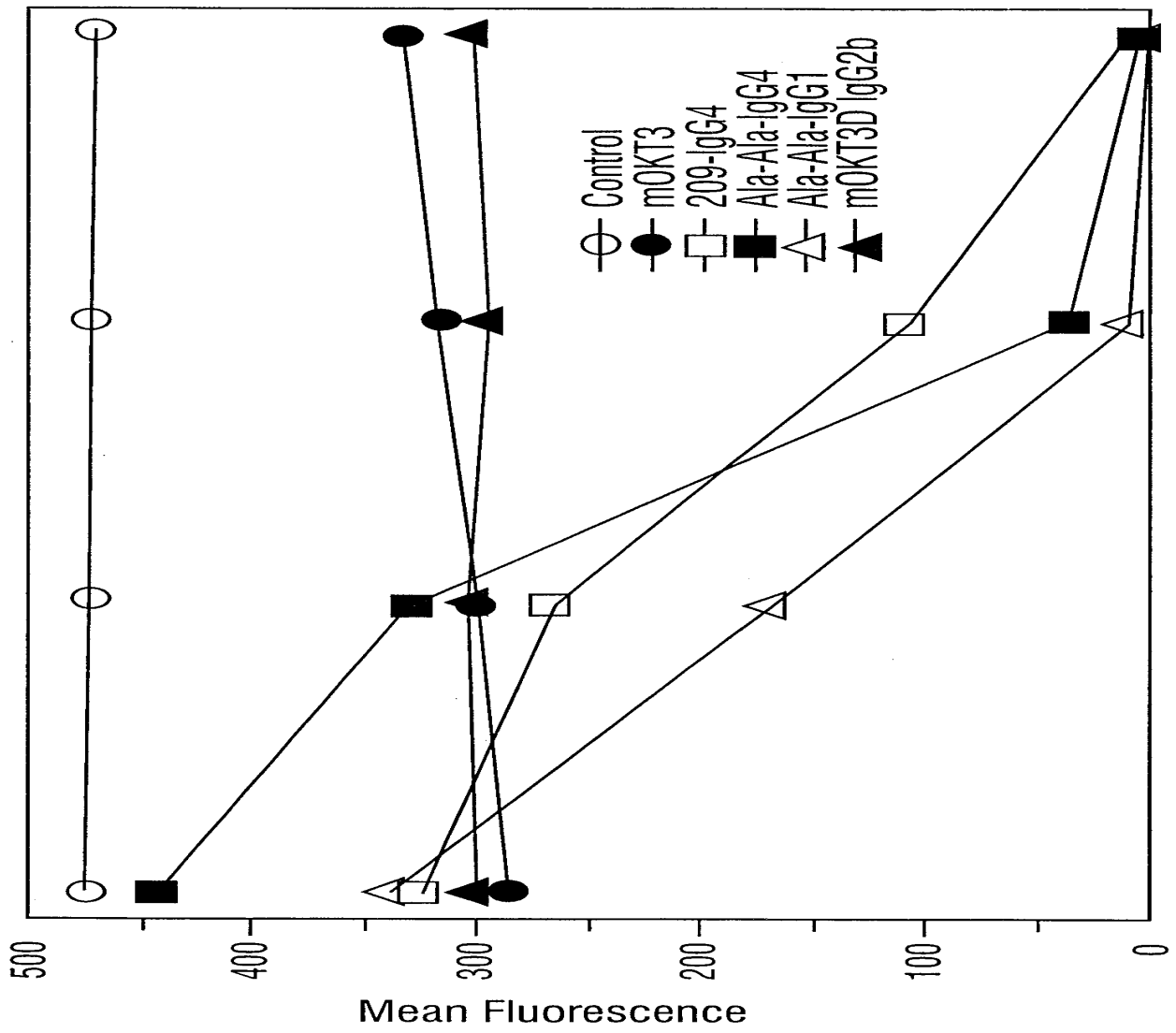
Control
mOKT3
209-IgG4
Ala-Ala-IgG4
Ala-Ala-IgG1
mOKT3D IgG2b
Mean Fluorescence
500
400
300
200
100
0
0.01
0.1
1
10
[mAb] (ug/ml)

FIG. 12A

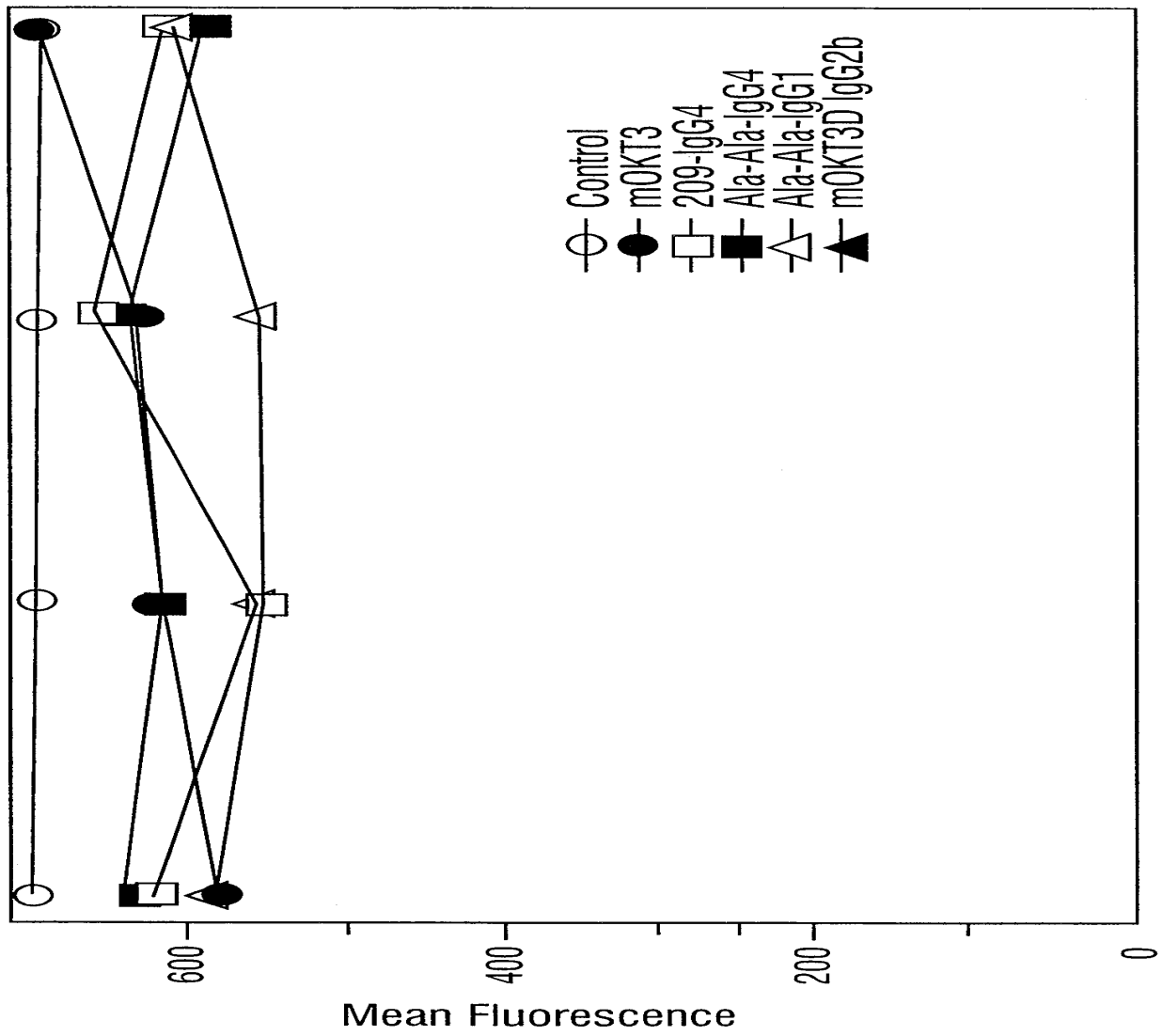
Control
mOKT3
209-IgG4
Ala-Ala-IgG4
Ala-Ala-IgG1
mOKT3D IgG2b
600
400
200
0
Mean Fluorescence
0.01
0.1
1
10
[mAb] (ug/ml)

FIG. 12B

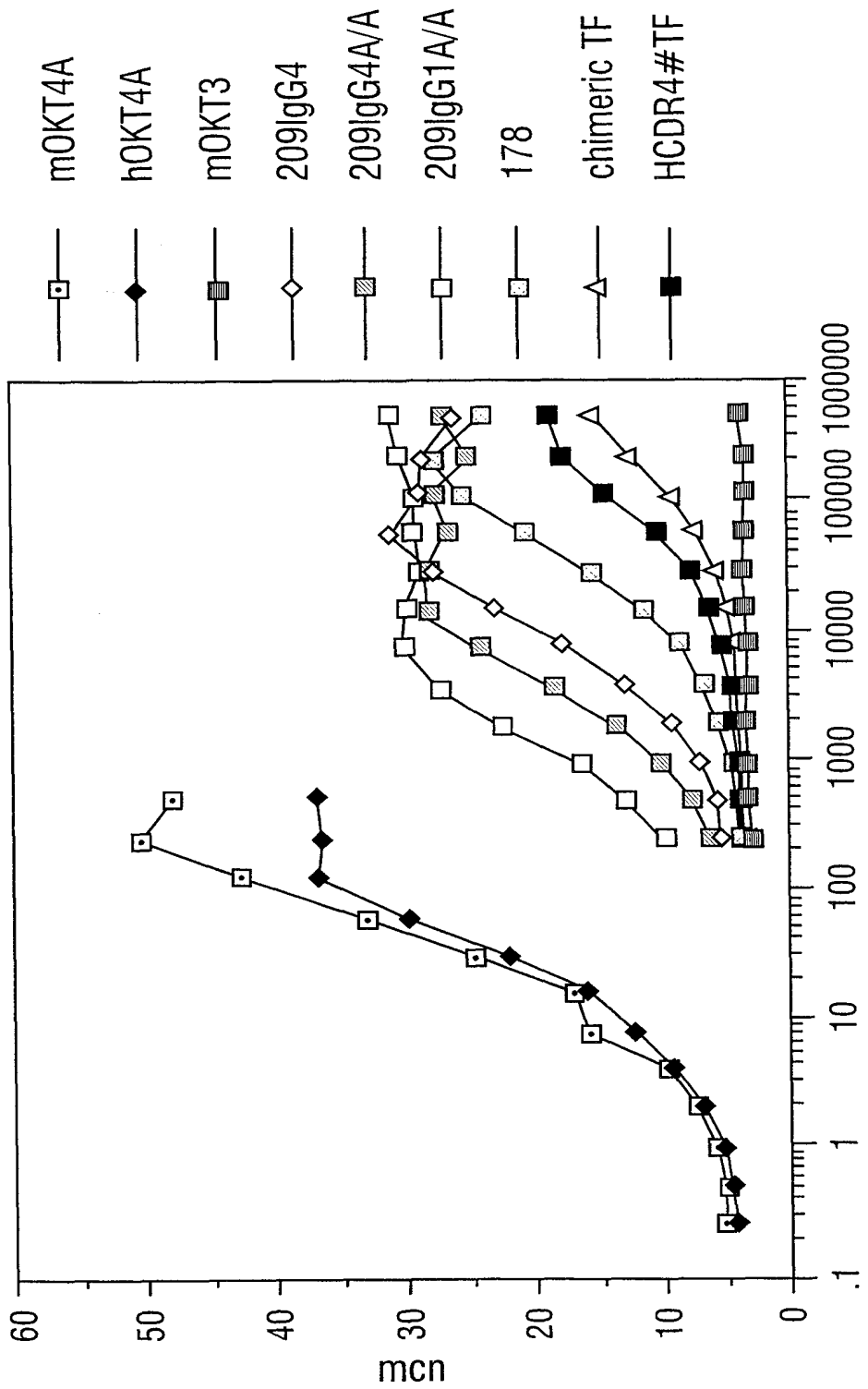
mOKT4A
hOKT4A
mOKT3
209IgG4
209IgG4A/A
209IgG1A/A
178
chimeric TF
HCDR4#TF
mcn
0
10
20
30
40
50
60
.1
1
10
100
1000
10000
100000
1000000
Ab conc. ng/ml

FIG. 13

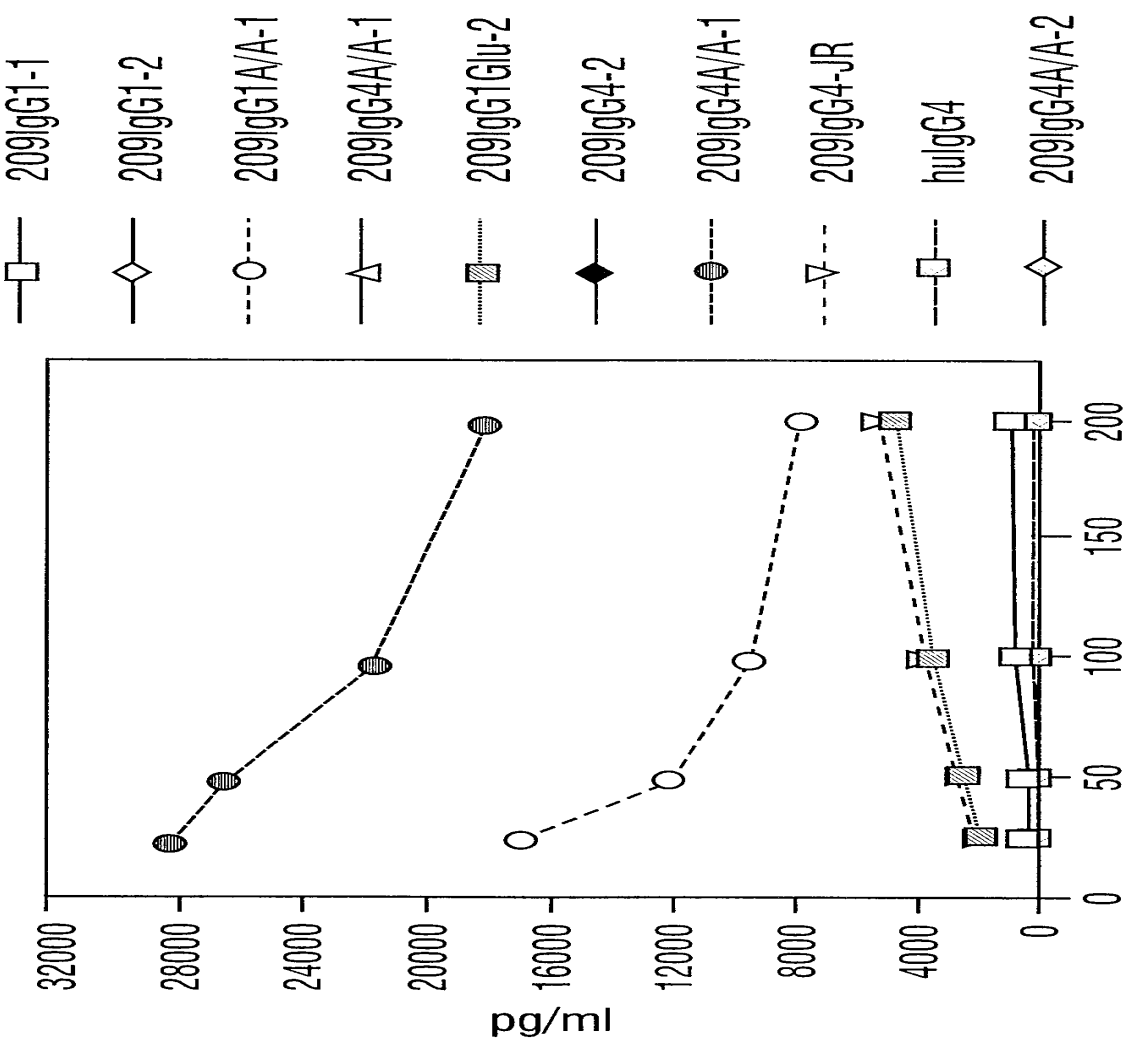
209IgG1-1
209IgG1-2
209IgG1A/A-1
209IgG4A/A-1
209IgG1Glu-2
209IgG4-2
209IgG4A/A-1
209IgG4-JR
huIgG4
209IgG4A/A-2
32000
28000
24000
20000
16000
12000
8000
4000
0
pg/ml
0
50
100
150
200
Ab conc. ug/ml

FIG. 14

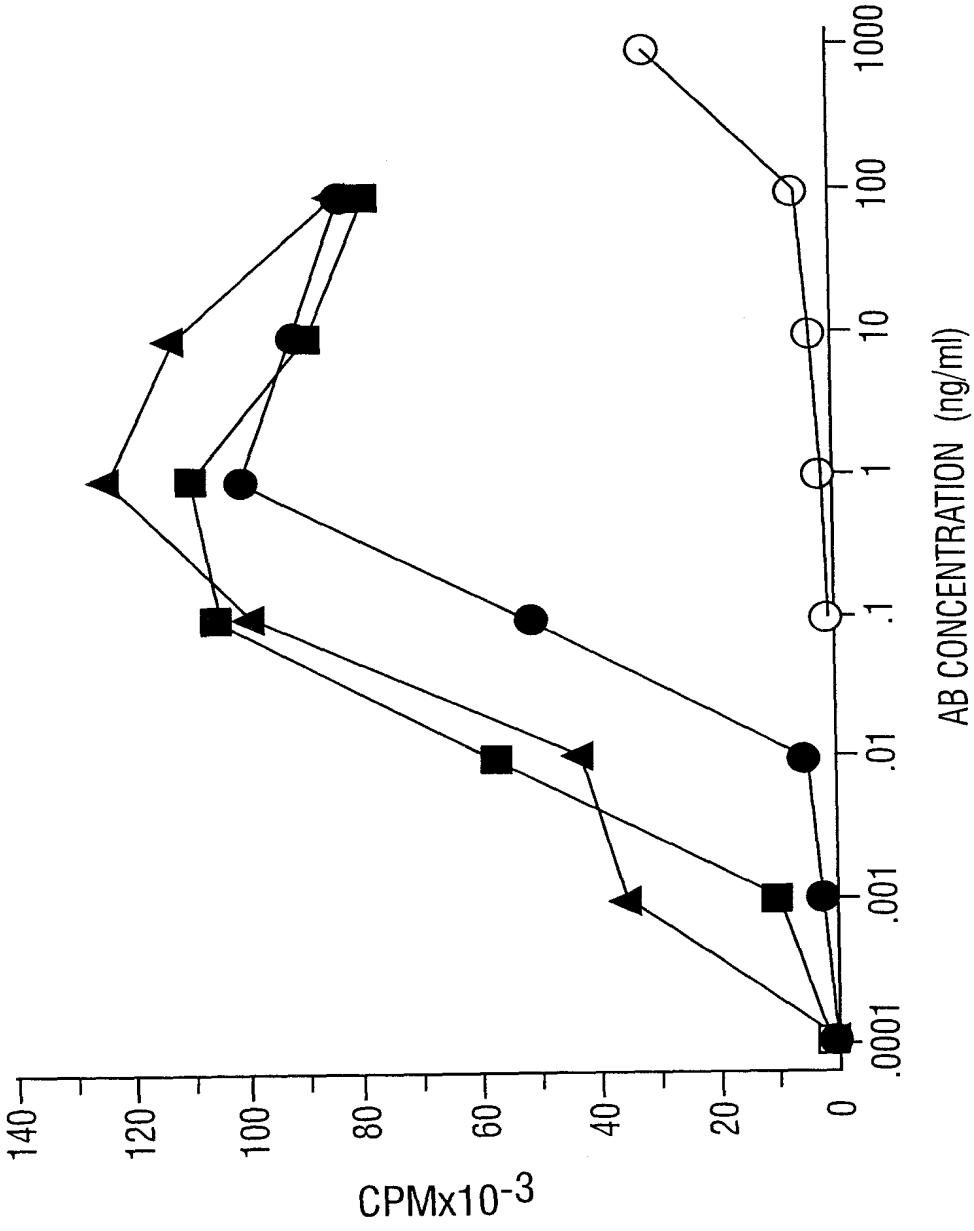
CPMx10-3
140
120
100
80
60
40
20
0
.0001
.001
.01
.1
1
10
100
1000
AB CONCENTRATION (ng/ml)

FIG. 15

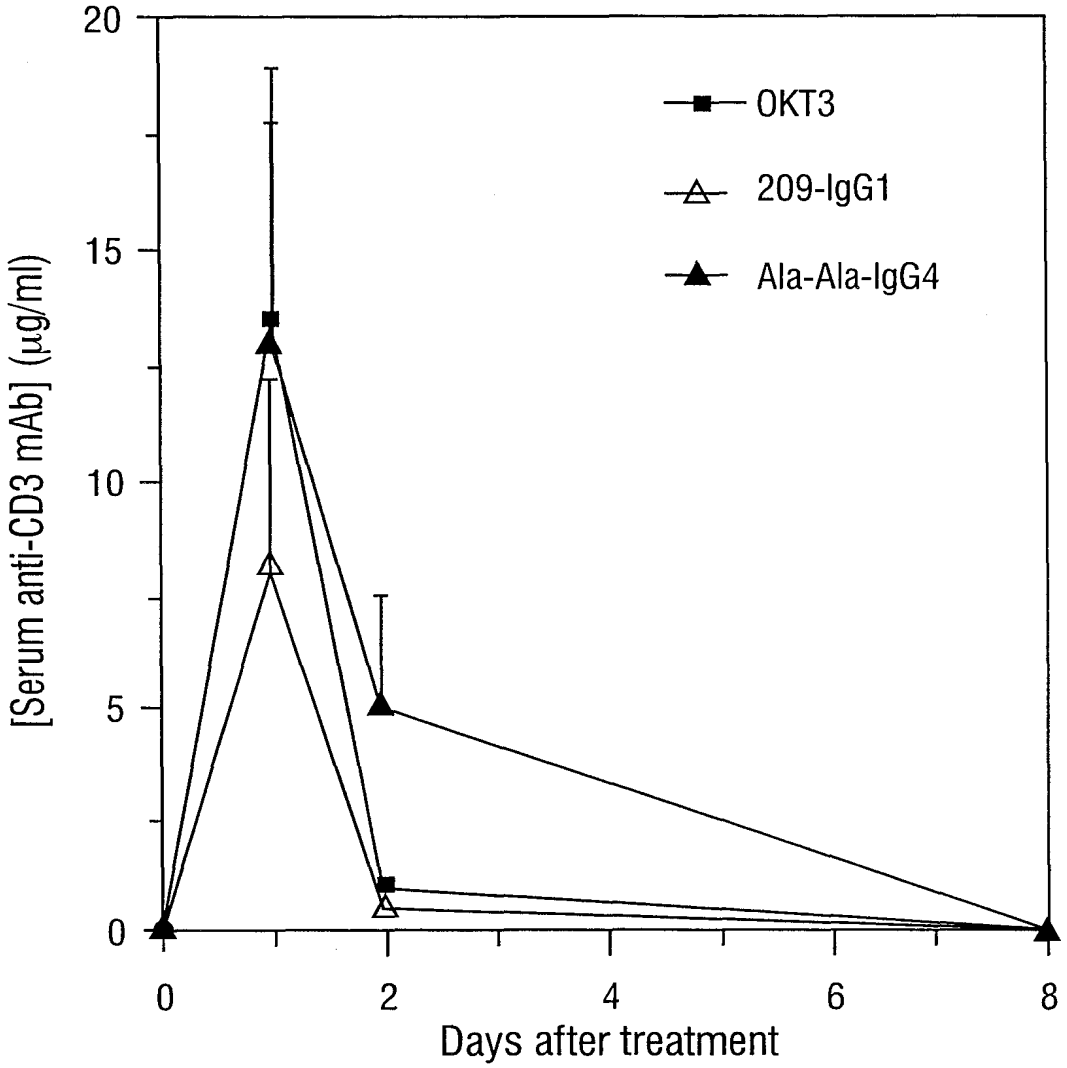
20
15
10
5
0
[Serum anti-CD3 mAb] (μg/ml)
OKT3
209-IgG1
Ala-Ala-IgG4
0
2
4
6
8
Days after treatment

FIG. 16

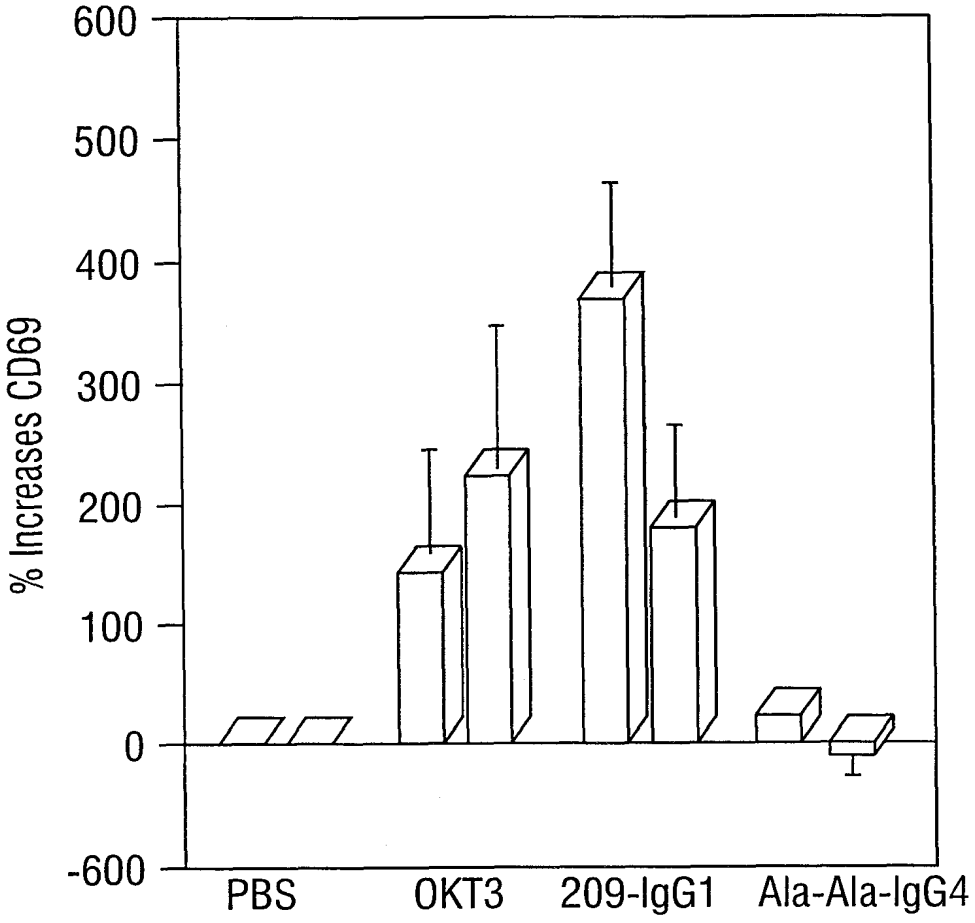
600
500
400
300
200
100
0
-600
% Increases CD69
PBS
OKT3
209-IgG1
Ala-Ala-IgG4

FIG. 17

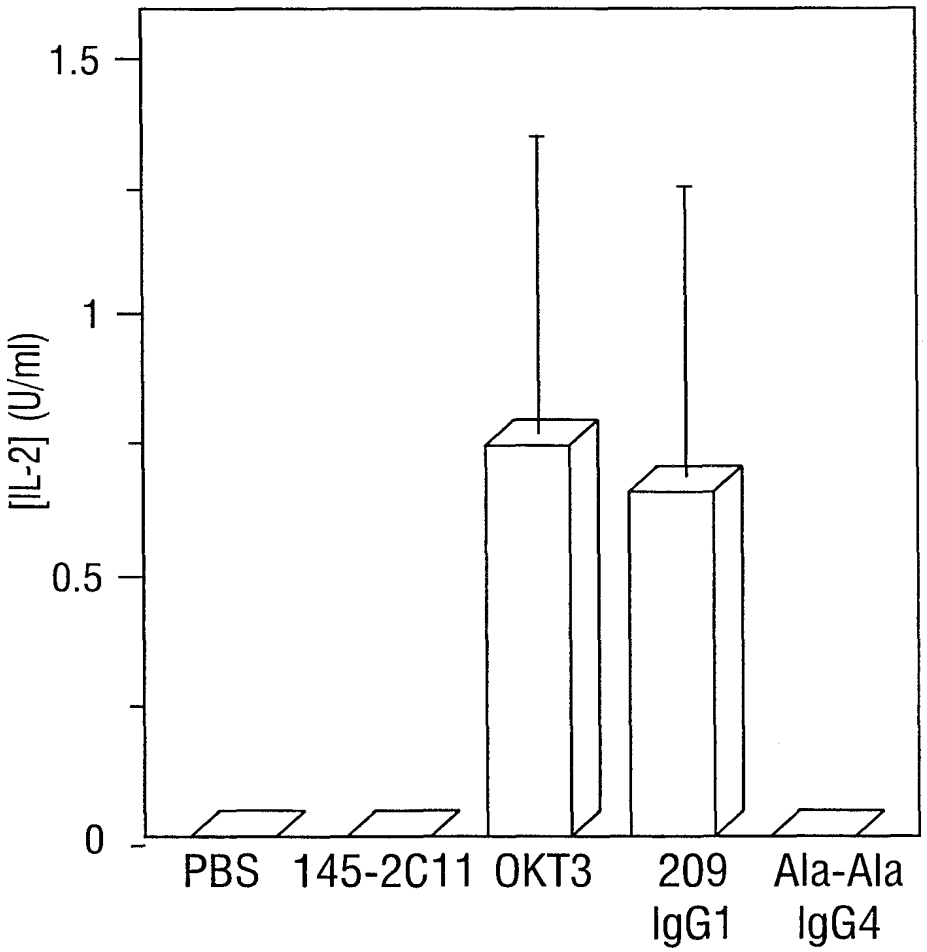

FIG. 18

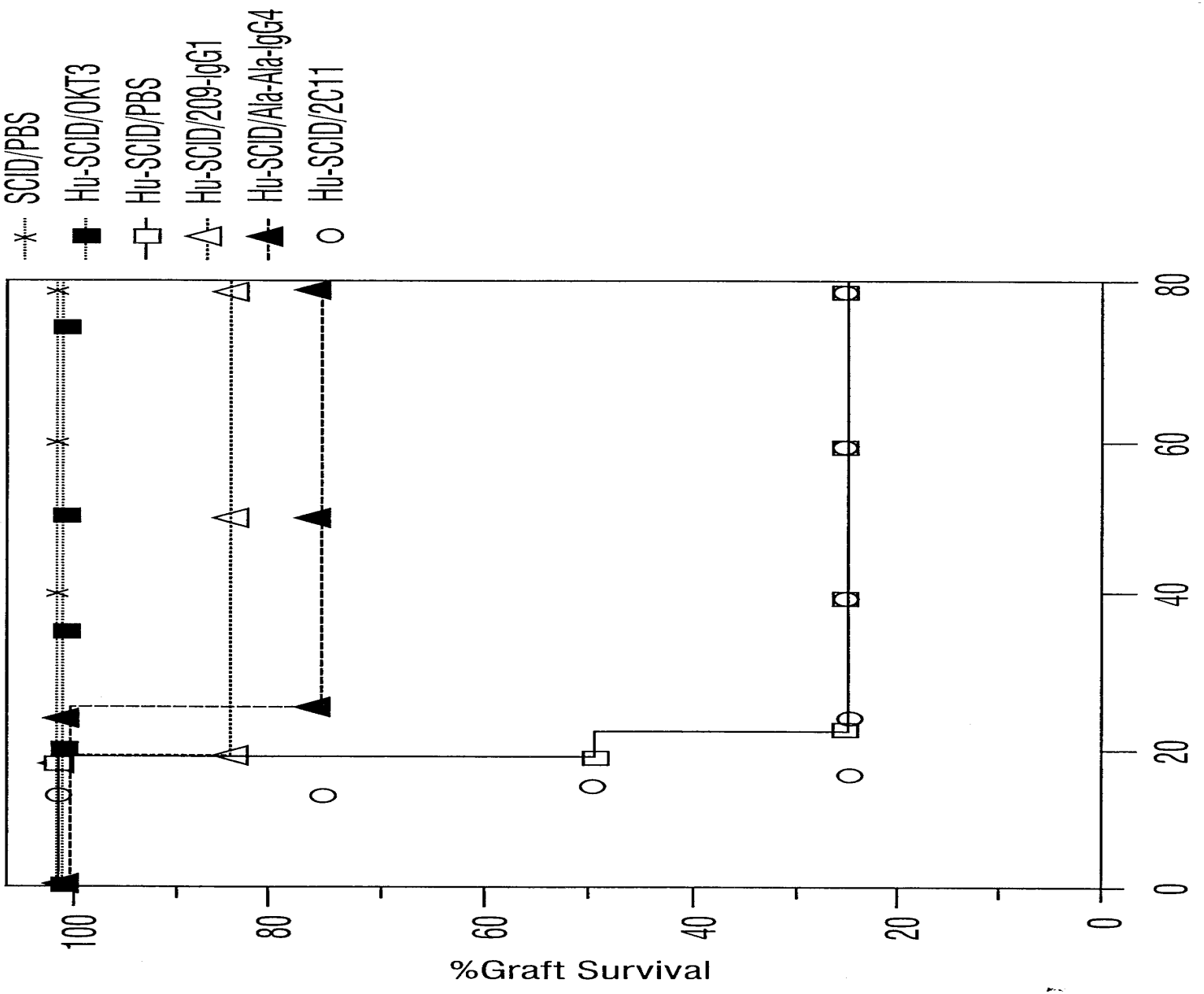
SCID/PBS
Hu-SCID/OKT3
Hu-SCID/PBS
Hu-SCID/209-IgG1
Hu-SCID/Ala-Ala-IgG4
Hu-SCID/2C11
100
80
60
40
20
0
%Graft Survival
0
20
40
60
80

FIG. 19

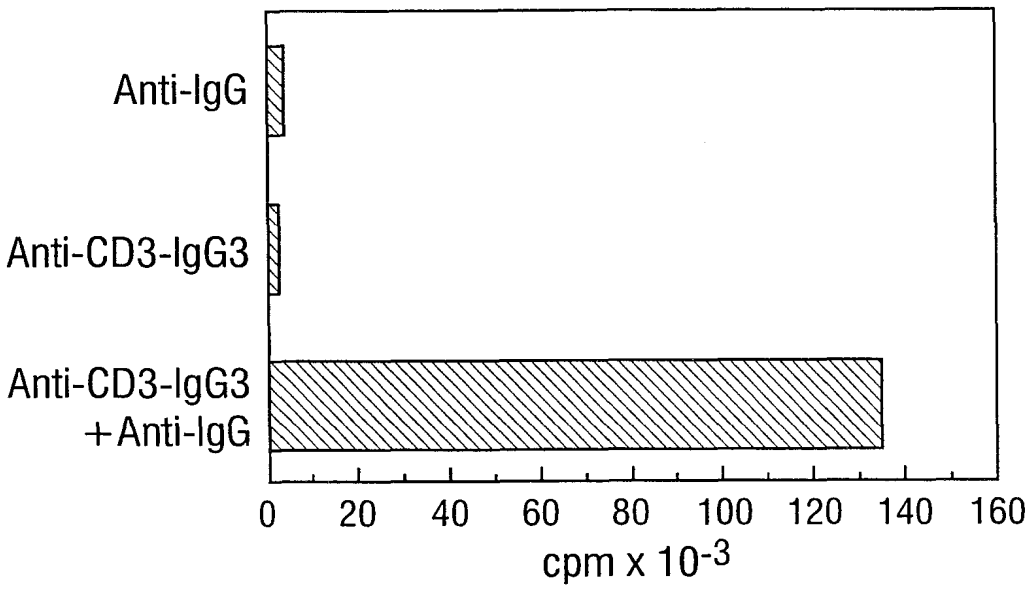

FIG. 20A

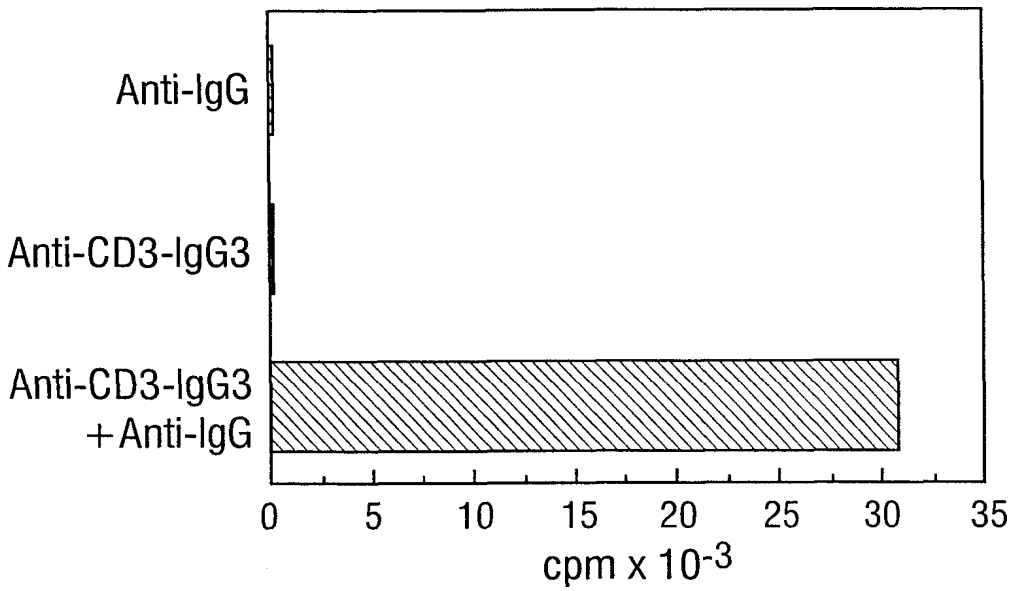

FIG. 20B

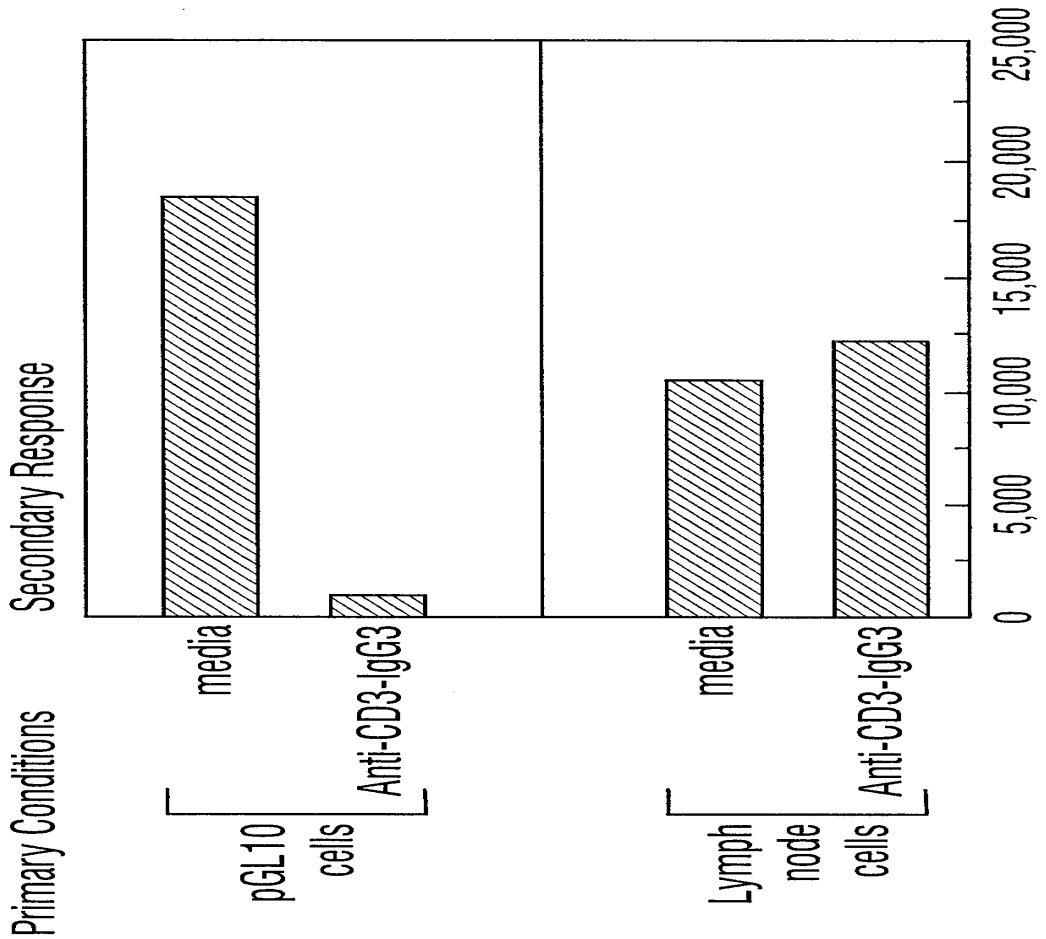
Primary Conditions
Secondary Response
pGL10 cells
media
Anti-CD3-IgG3
Lymph node cells
media
Anti-CD3-IgG3
0
5,000
10,000
15,000
20,000
25,000
cpm

FIG. 21A

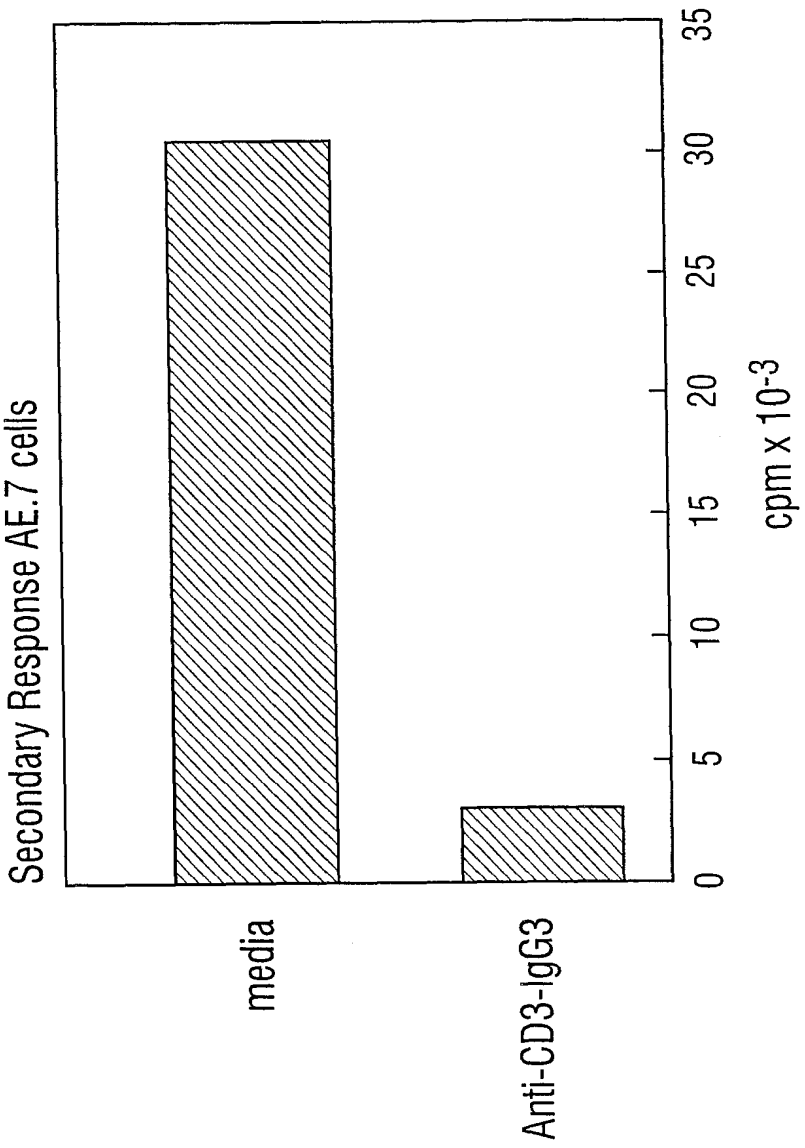
Secondary Response AE.7 cells
0
5
10
15
20
25
30
35
cpm x 10-3
media
Anti-CD3-IgG3

FIG. 21B

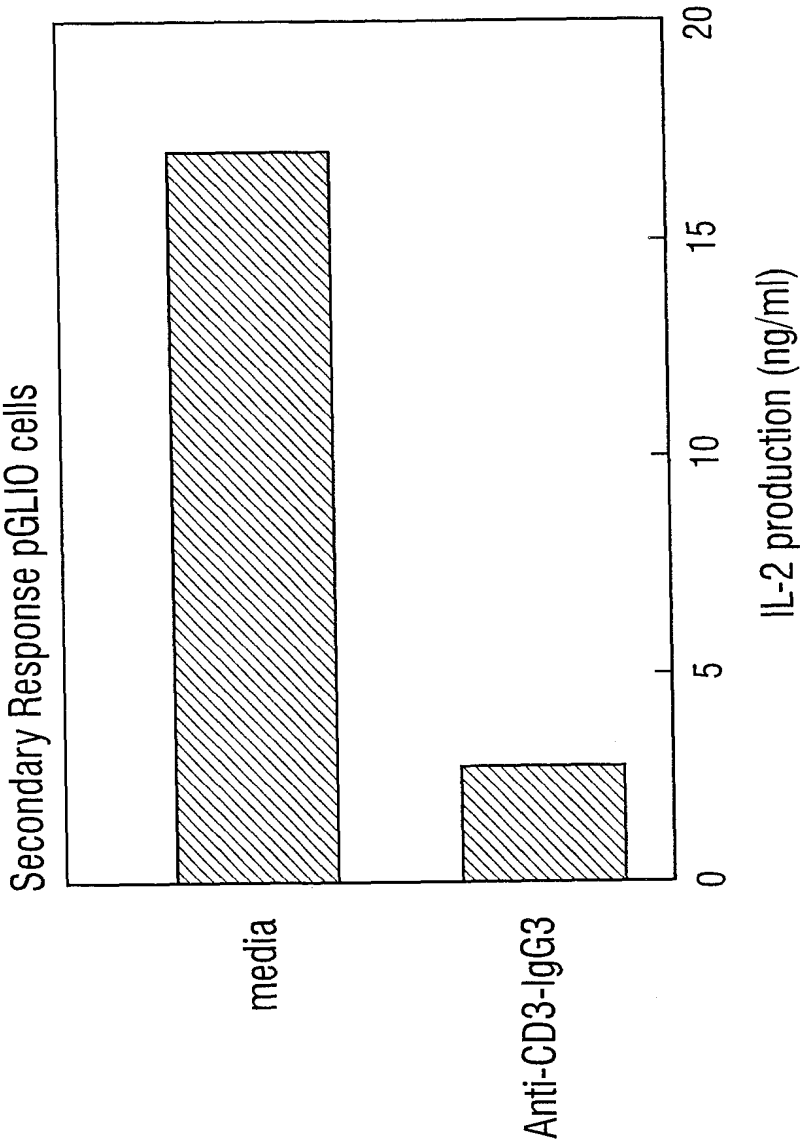
Secondary Response pGL10 cells
0
5
10
15
20
IL-2 production (ng/ml)
media
Anti-CD3-IgG3

FIG. 21C

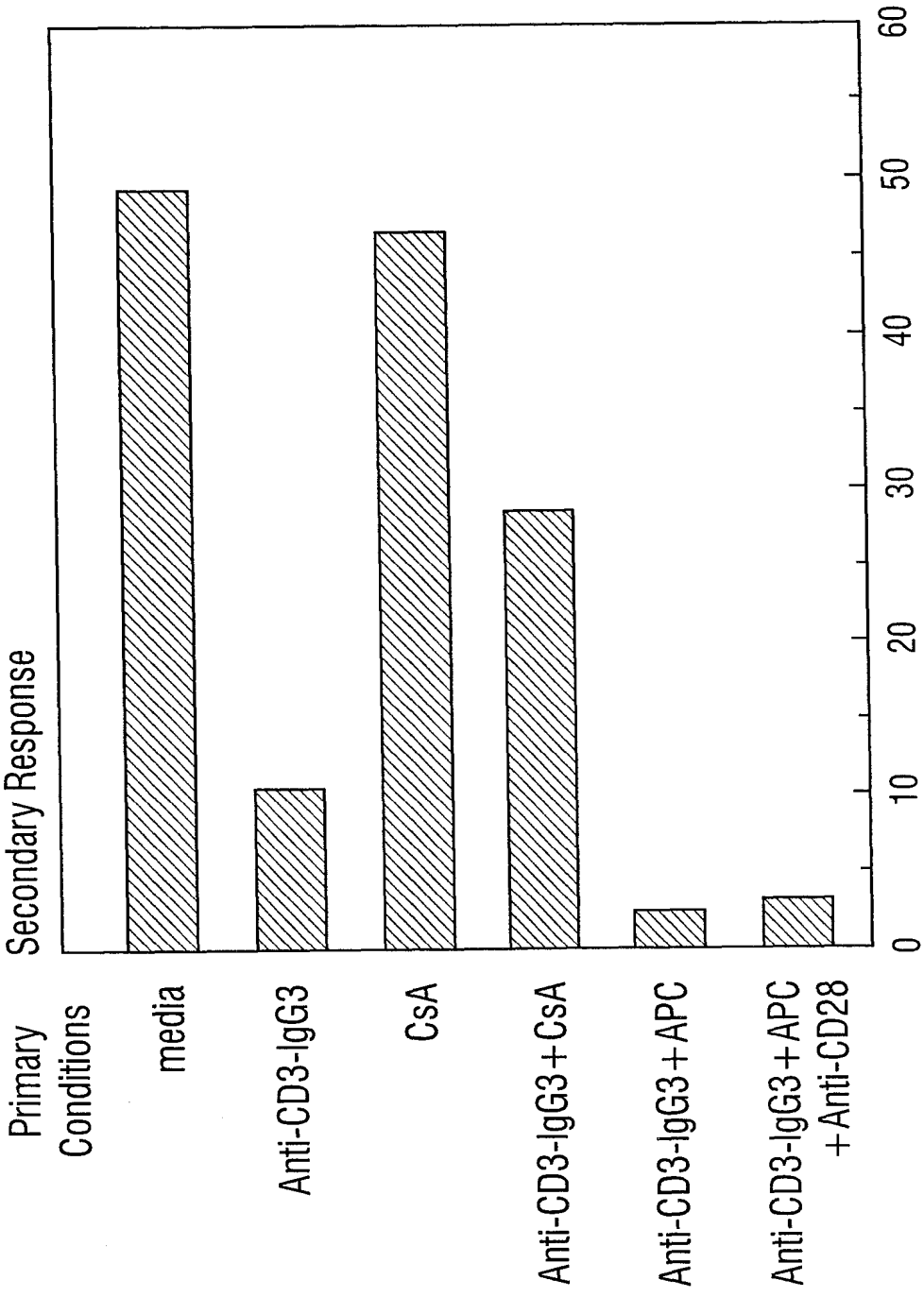
Secondary Response
Primary Conditions
media
Anti-CD3-IgG3
CsA
Anti-CD3-IgG3+CsA
Anti-CD3-IgG3+APC
Anti-CD3-IgG3+APC +Anti-CD28
0
10
20
30
40
50
60
cpm x 10-3

FIG. 21D

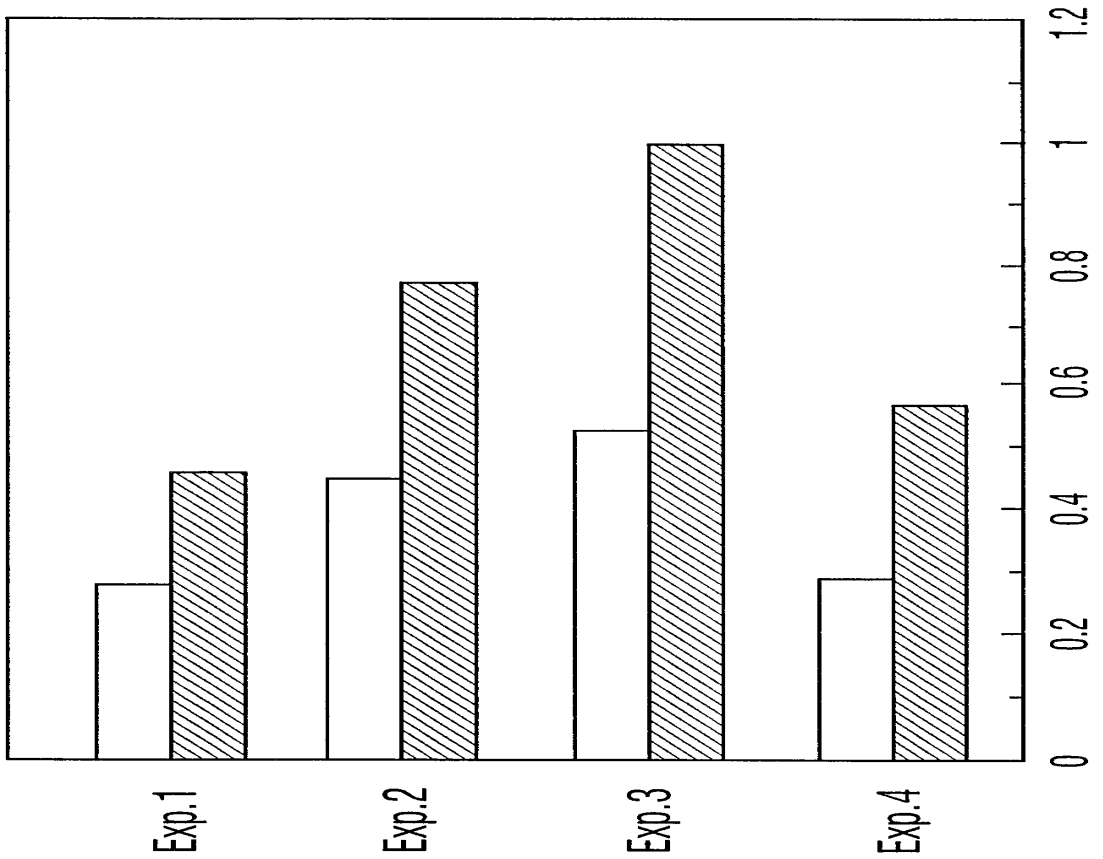

FIG. 22

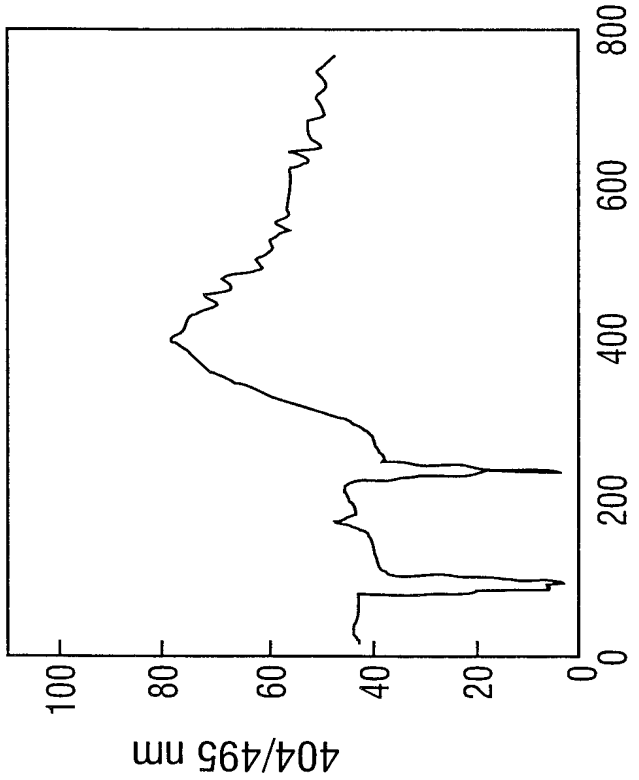

FIG. 23B

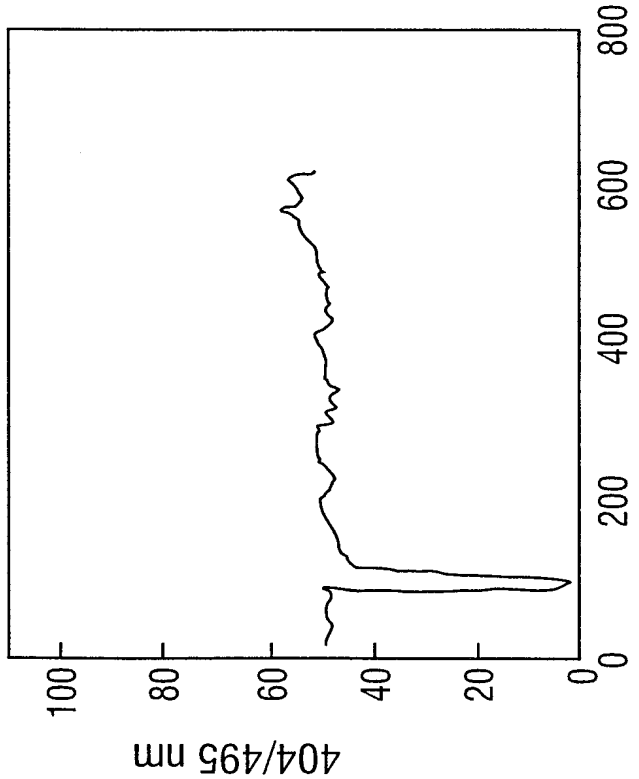

FIG. 23A

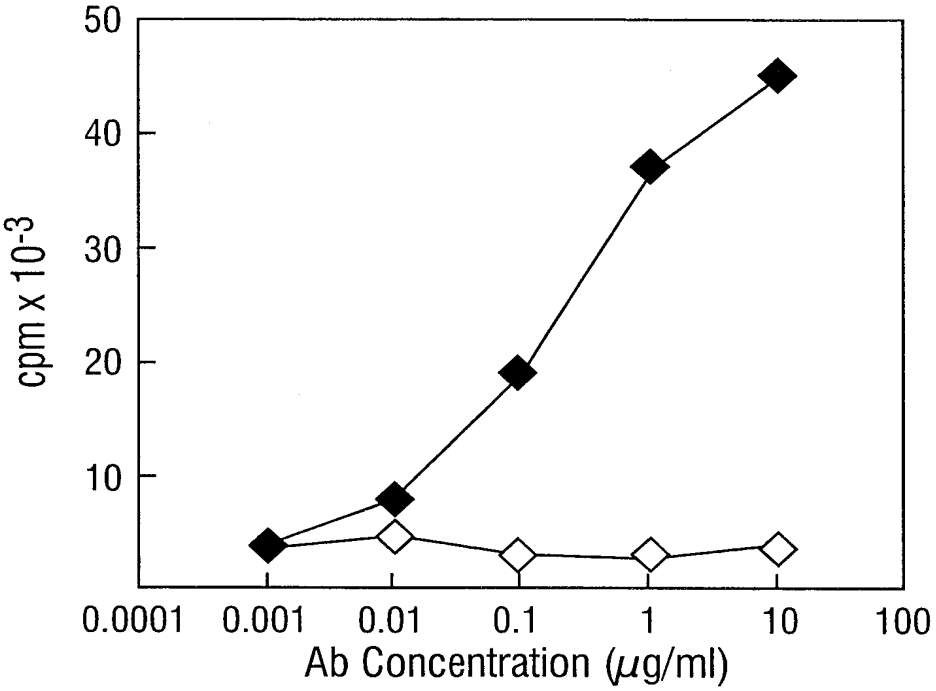

FIG. 24A

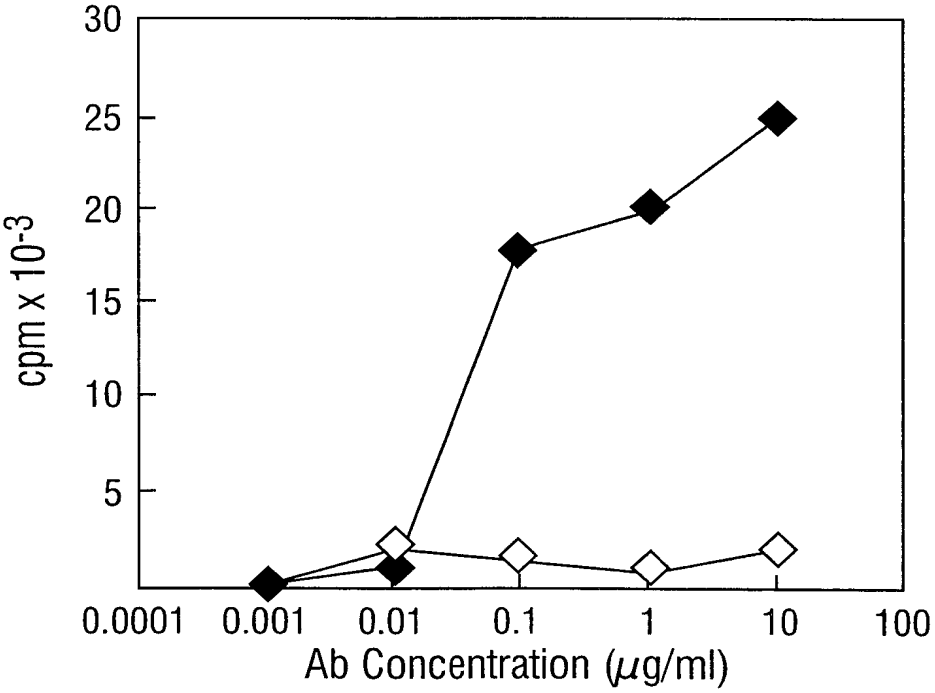

FIG. 24B

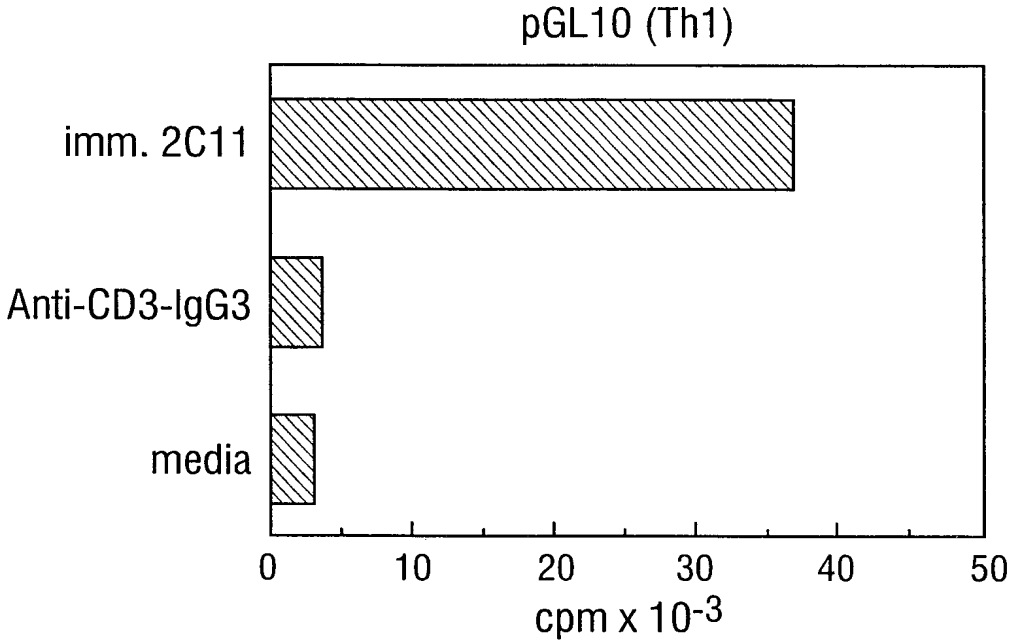

FIG. 25A

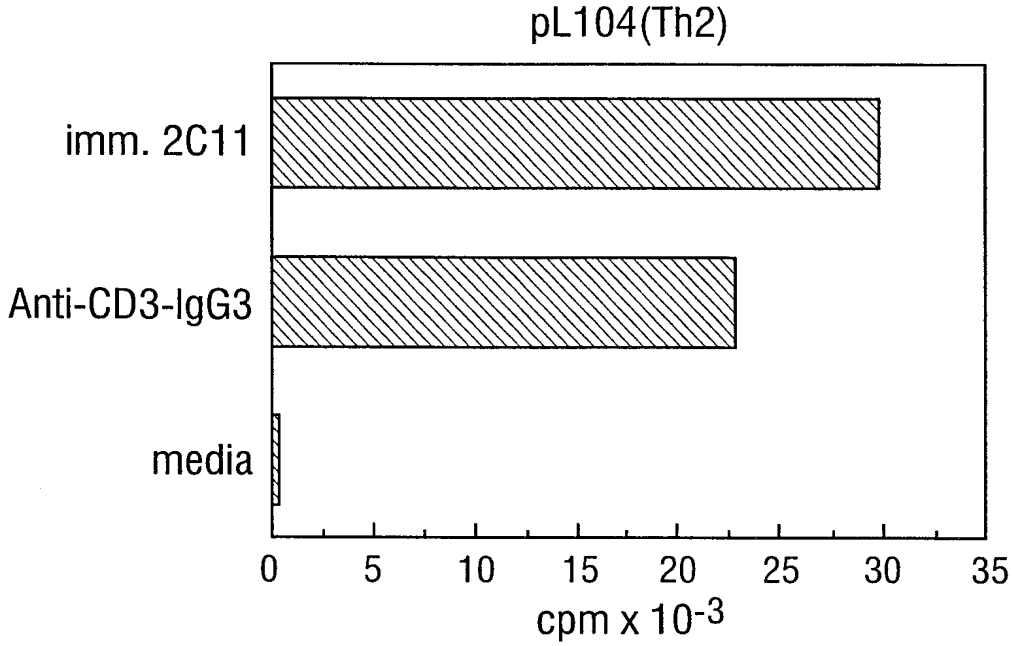

FIG. 25B

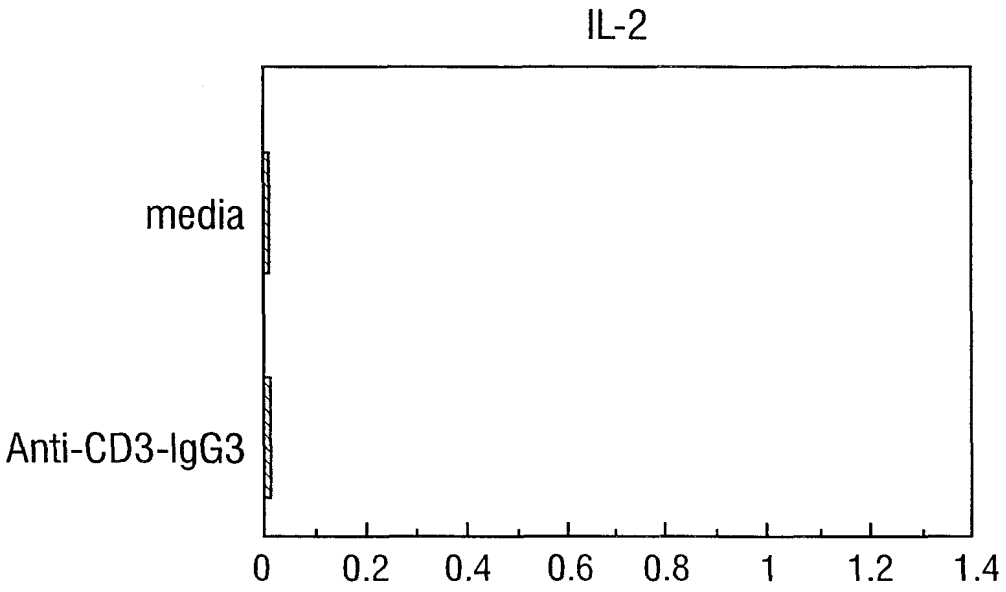

FIG. 26A

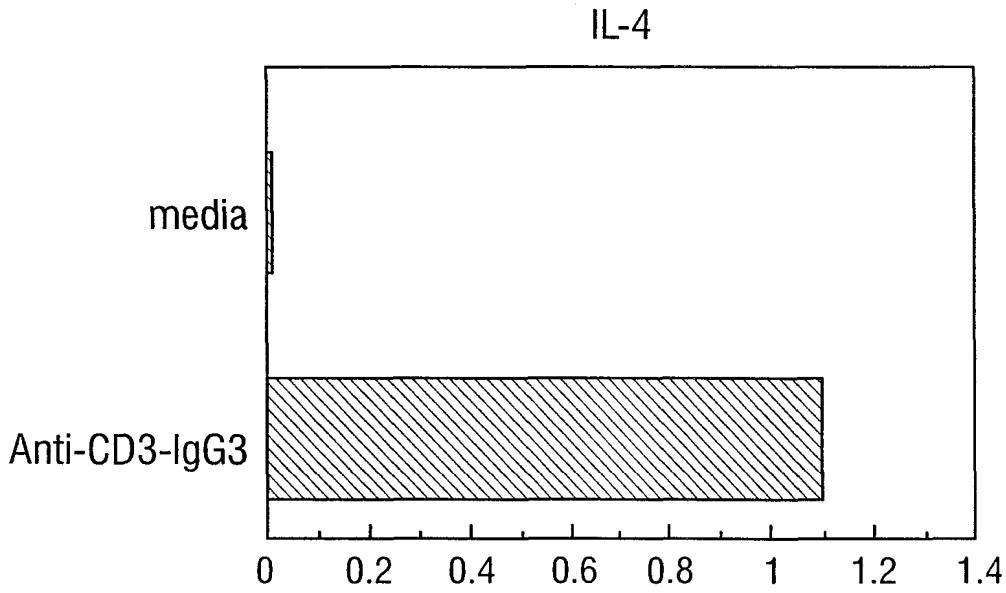

FIG. 26B

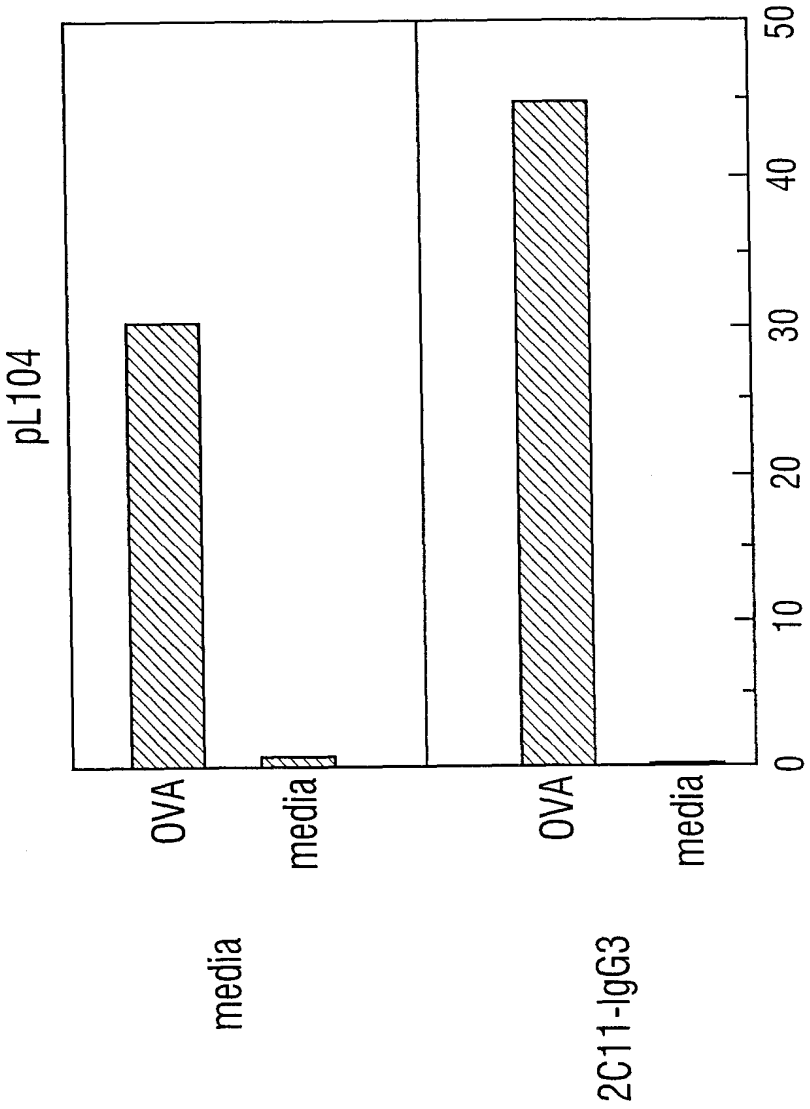

FIG. 27

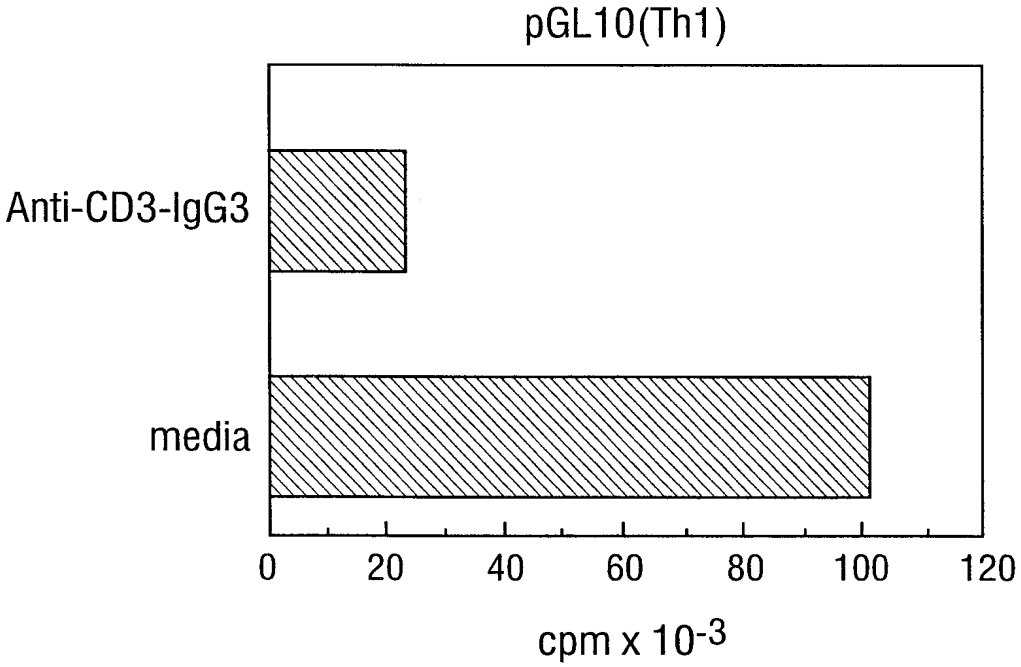

FIG. 28A

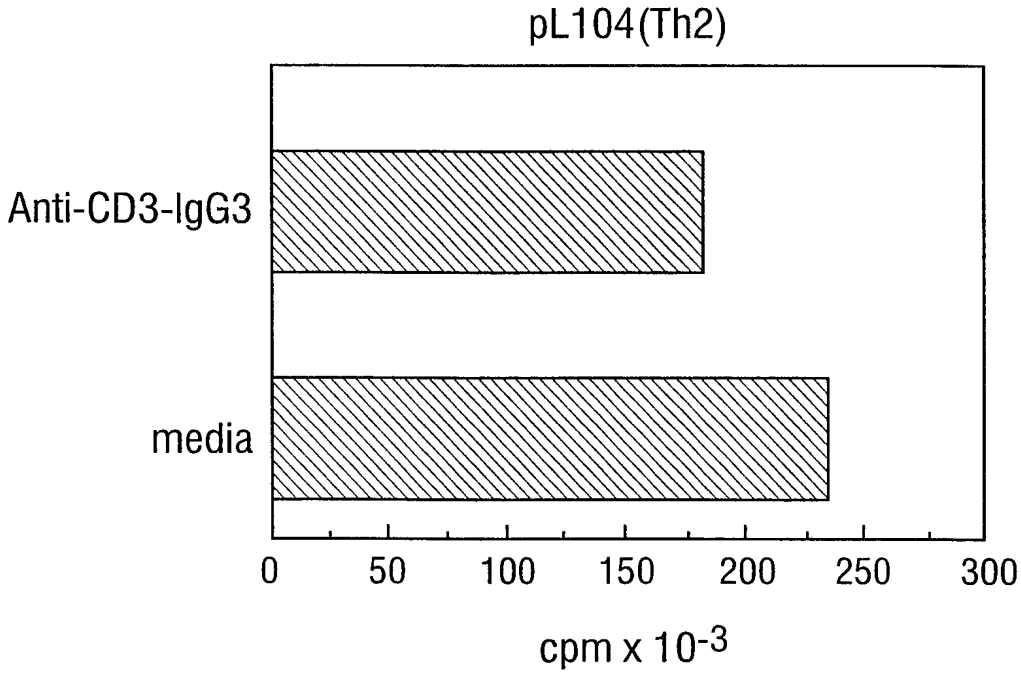

FIG. 28B

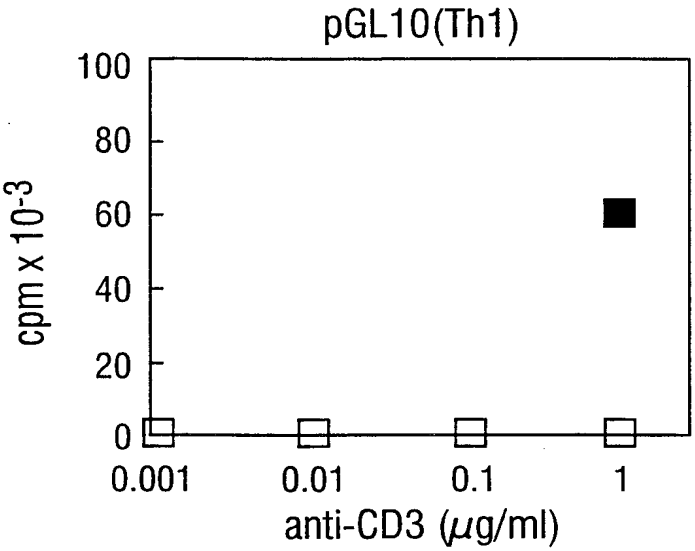

FIG. 29A

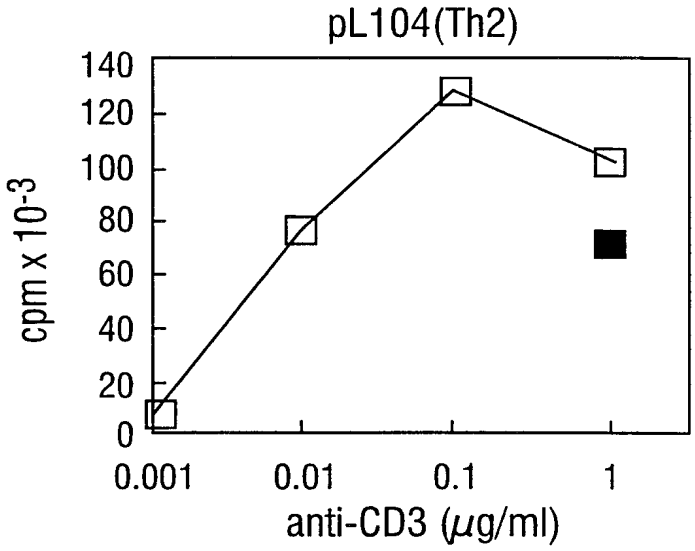

FIG. 29B

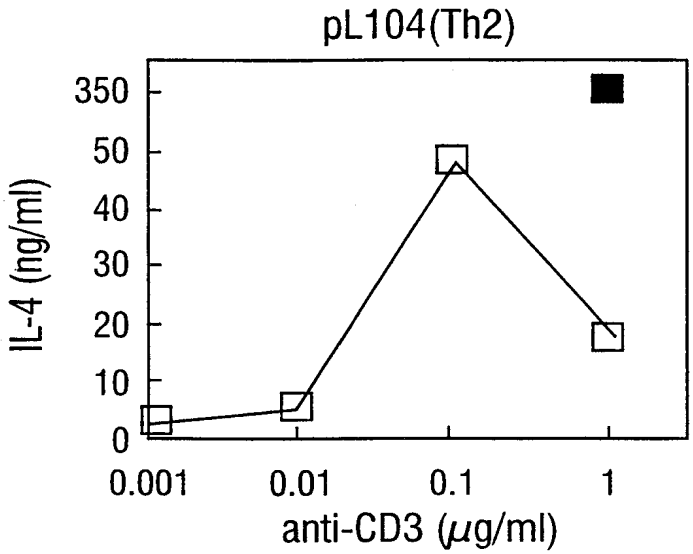

FIG. 29C

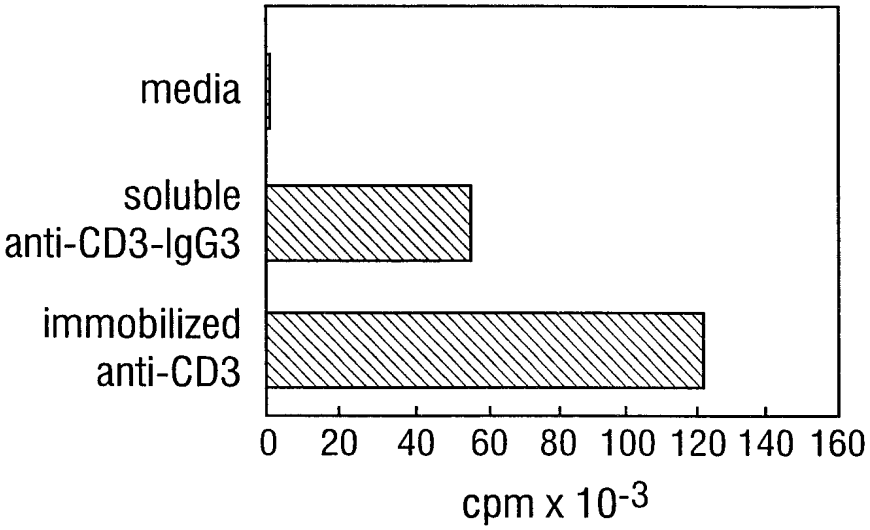

FIG. 30A

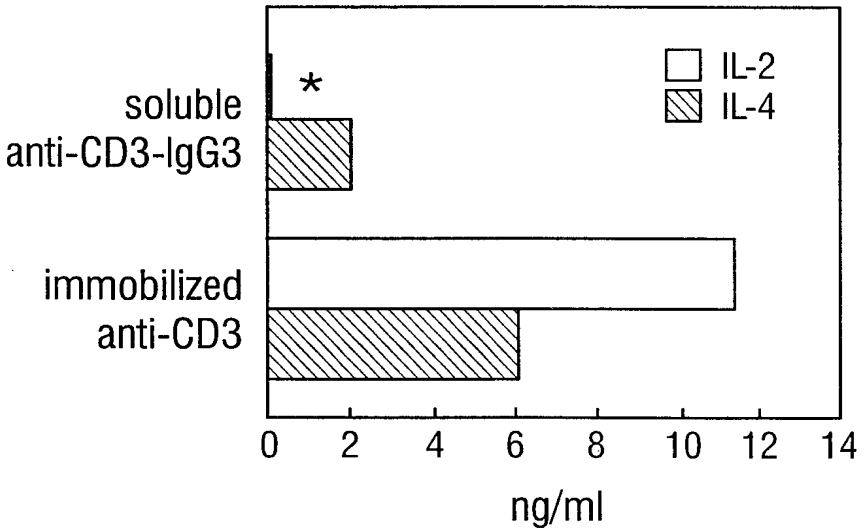

FIG. 30B

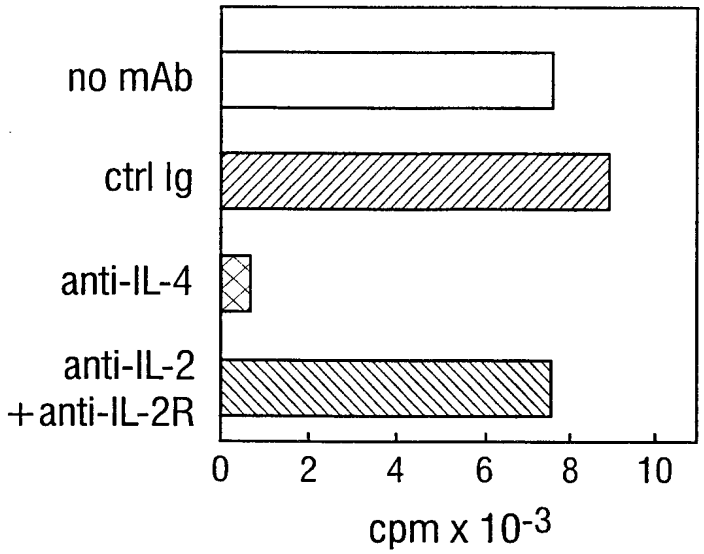

FIG. 31A

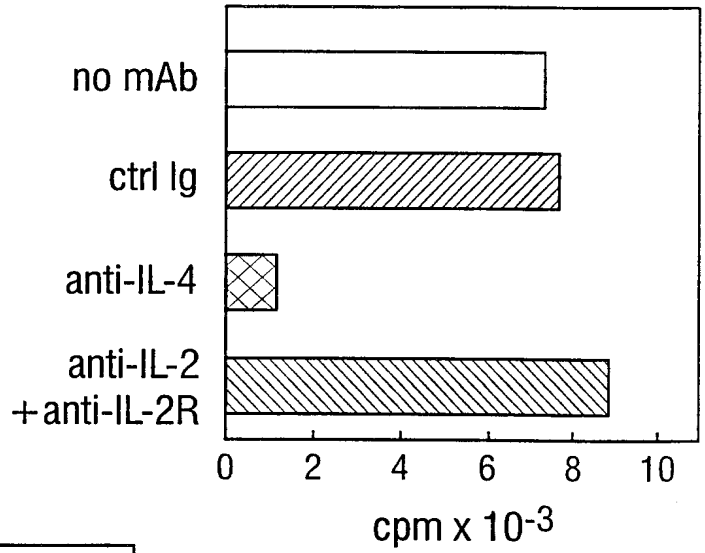

FIG. 31B

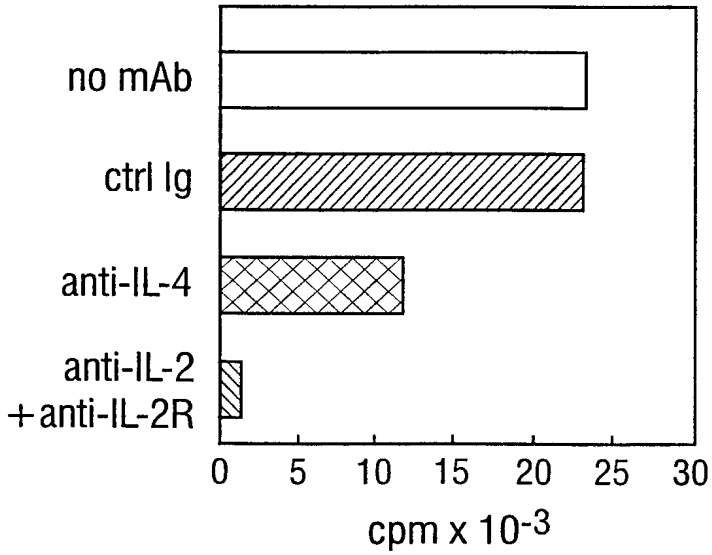

FIG. 31C

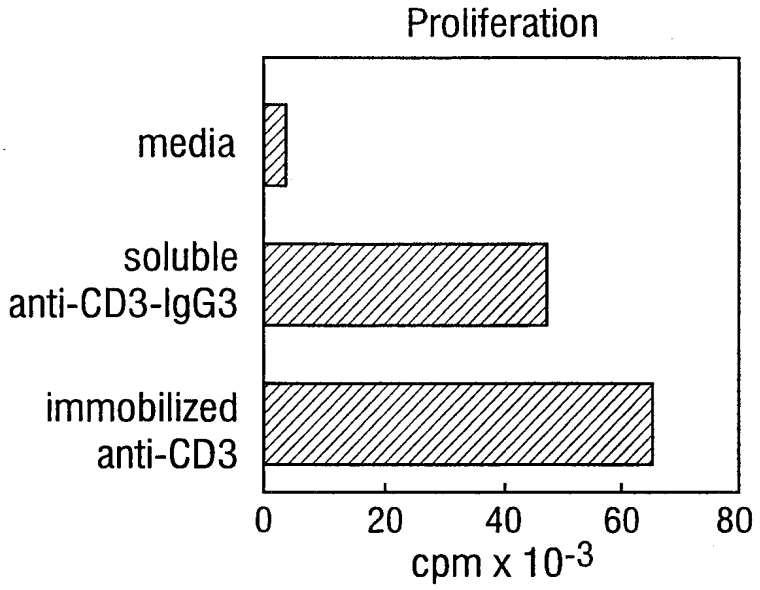

FIG. 32A

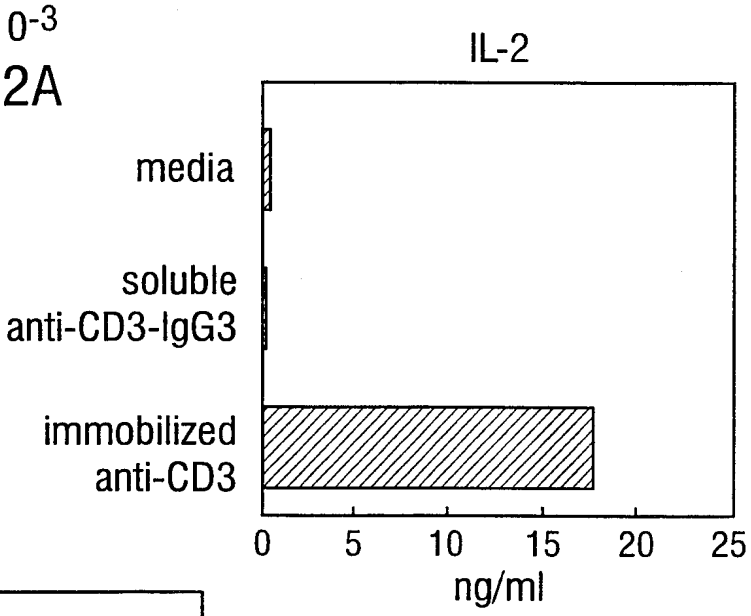

FIG. 32B

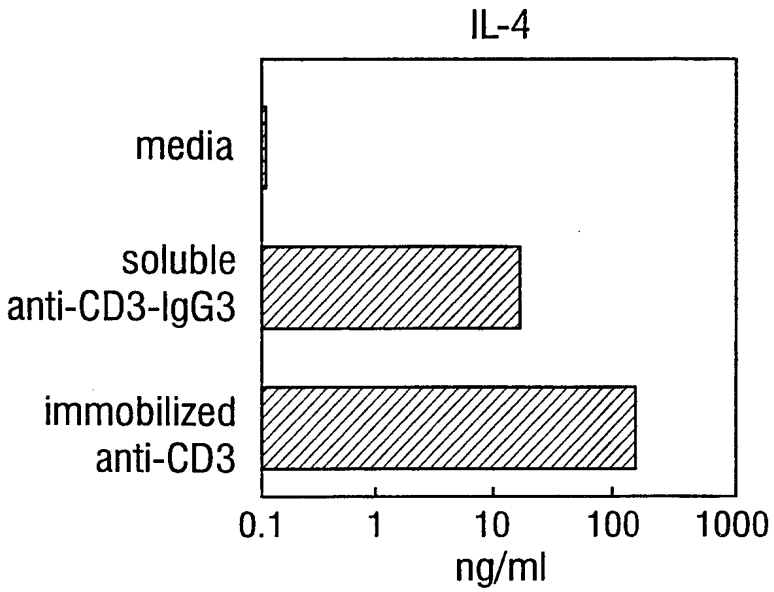

FIG. 32C

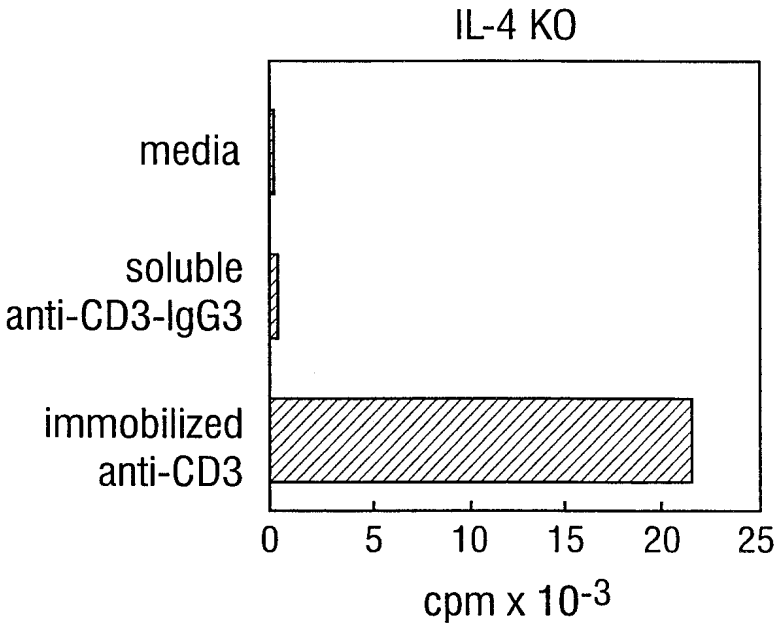

FIG. 33A

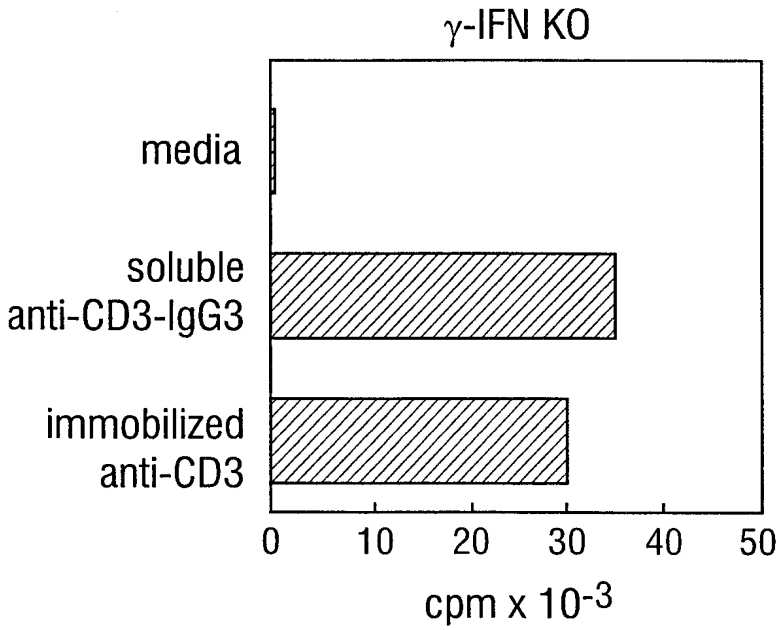

FIG. 33B

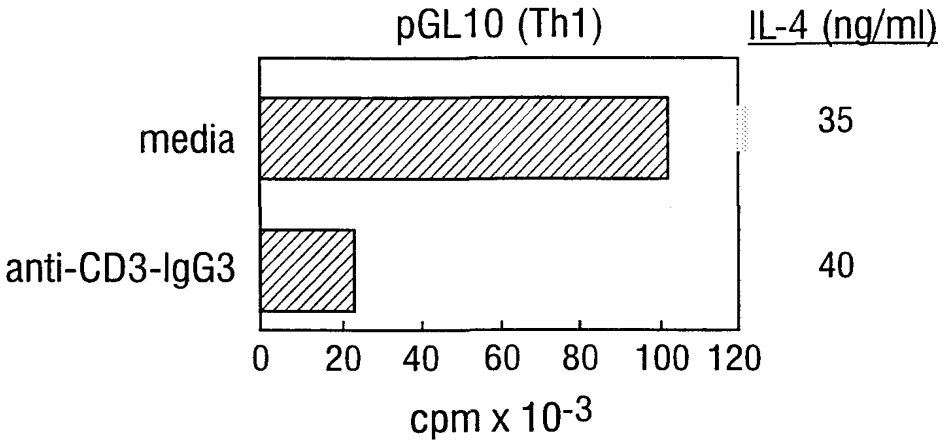

FIG. 34A

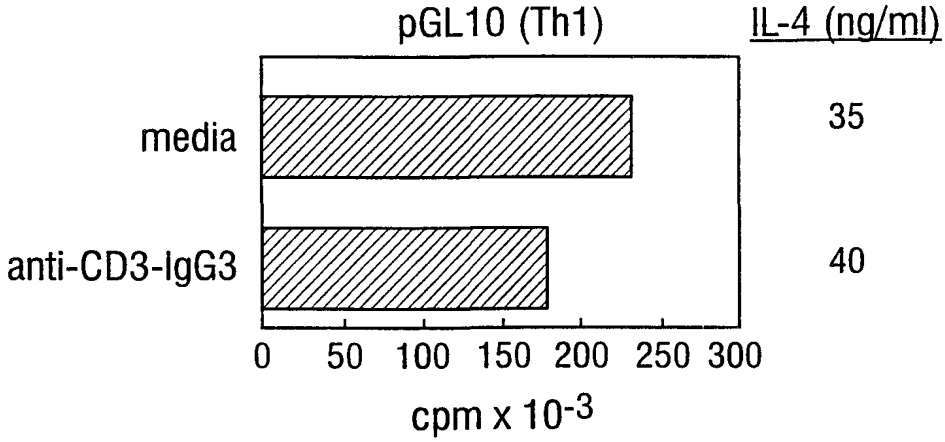

FIG. 34B

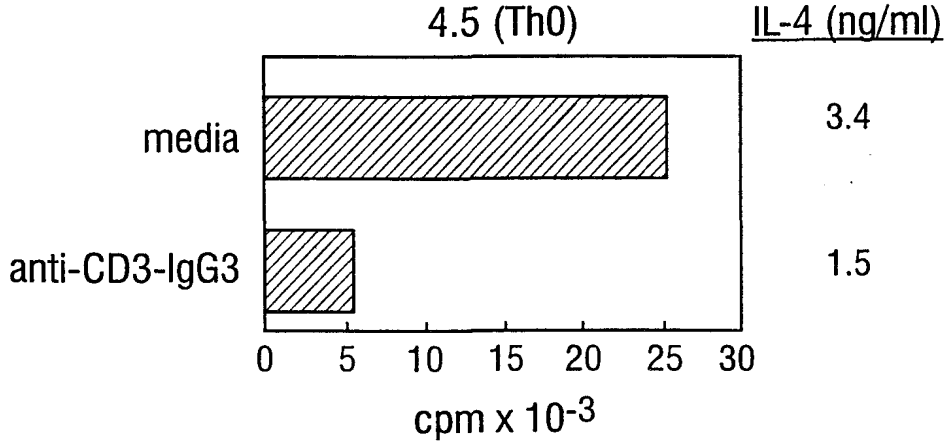

FIG. 34C

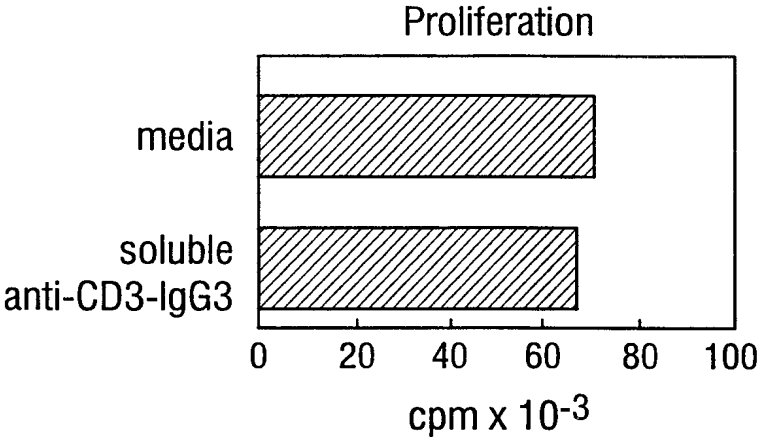

FIG. 35A

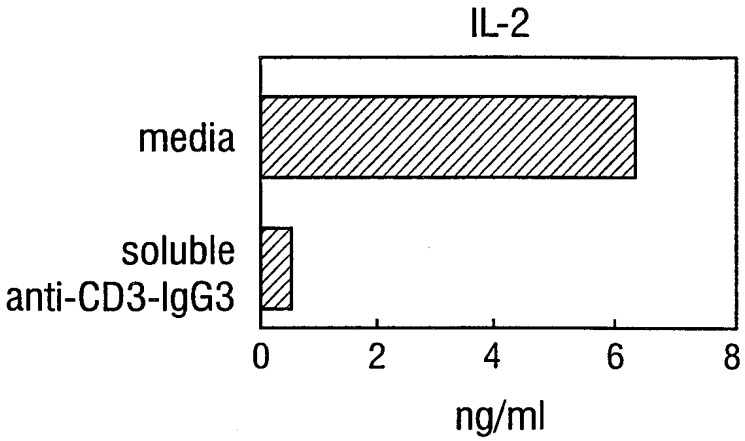

FIG. 35B

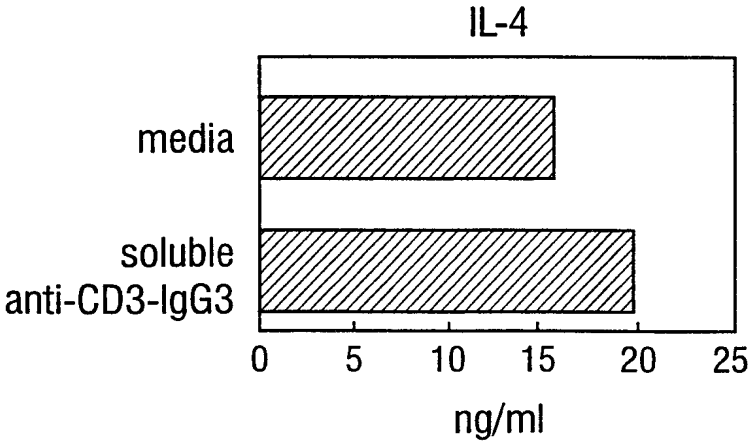

FIG. 35C

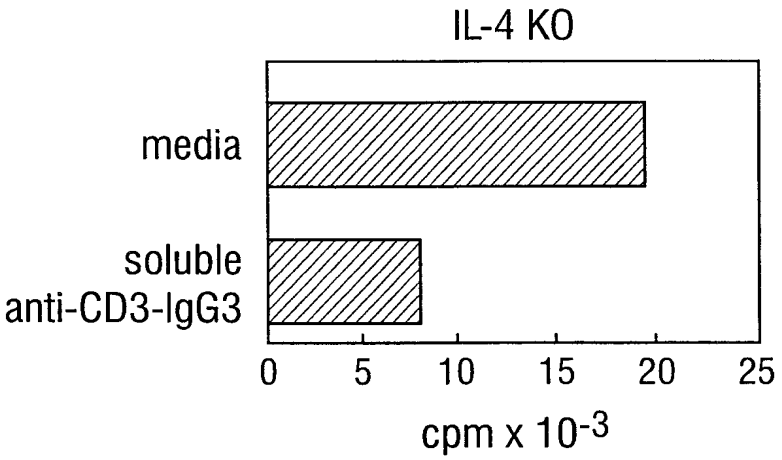

FIG. 36A

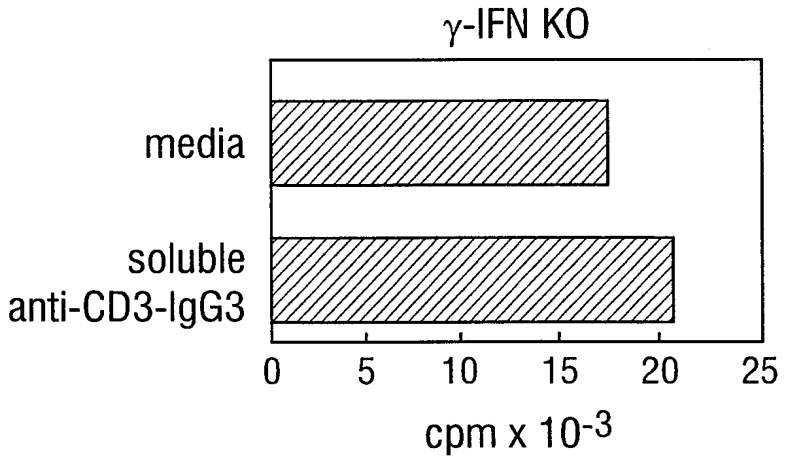

FIG. 36B